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(54) Title: BINDING PROTEIN

FLT 316 GPSFKSVNTSVHIY 330DKAFITVKHRKQQVLE-TVAGKRSYRLSMKVKA
KDR 312 GLMTKKNSTFVRVH 326EKPVAFGSGMESLVEATV-GER-VRI
FLK 314 GRMIKRNRRTFVRVH 328TKPPIAFGSGMKSLVEATV-GSQ-VRI
FLT4 315 GIQRFRESTEVIVH 329BNPPISVEWLKGPILEATA-GDBLVKL
PDKYKDG----KALSGRHs

FLT GYSLIIKDVTEEDAGNYTILL--SI---KQSNVFKNLATLIVNVKPOIYEKAVSSFPD 440 PALYPLG447
KDR GHVLTIMEVSERDTGNYTVILTNPISKEKQSHVV----SLVVYVPPQIGEKSLISPVD 433 SYQY--G438
FLK GDELTIMEVTERDAGNYTVILTNPISMEKQSHMV----SLVVNVPPQIGEKALISPMD 435 SYQY--G440
FLT4 PHALVLKEVTEASTGTYTLALWNSAAGLR RNISLELVNVPPQIHEKEASSPS- 433 IYSR---437

Underlined:

Construct 0

(57) Abstract: The invention relates to novel compounds that act to prevent dimerisation of vascular endothelial growth factor (VEGF) receptors. The novel compounds may comprise the amino acid sequence of the fourth Ig-like domain of a VEGF receptor, or a variant that retains the ability to bind to a VEGF receptor. These compounds are useful in the inhibition of the biological activity of VEGF receptors and may thus be used to treat diseases in which VEGF plays a role.

WO 01/42284 A2

BINDING PROTEIN

The present invention relates to novel proteins that act to prevent dimerisation of vascular endothelial growth factor (VEGF) receptors. These proteins are useful in the inhibition of the biological activity of VEGF receptors and may thus be used to treat diseases in which

5 VEGF plays a role.

VEGF is a potent stimulator of angiogenesis and plays an important role in the mammalian body in the development of the vascular system. It has been implicated in various human diseases such as inflammation, psoriasis, rheumatoid arthritis, hemangiomas, diabetic retinopathy, angiofibromas, macular degeneration, endometriosis, retinal neovascularisation and cancer. The molecule has been implicated particularly in solid tumours, whose growth can be prevented by the inhibition of VEGF action (Kim *et al.*, 10 (1993) *Nature* 362: 841-844)

VEGF plays a role in endometriosis (McLaren *et al.*, (1996) *Human Reproduction* 11, No.1, 220-223; McLaren *et al.*, (1996) *J. Clin. Invest.* 98 No.2, 482-489), the name given 15 to the disease that results from the presence of endometrium outside the uterine cavity. This disease affects women during their childbearing years with deleterious social, sexual and reproductive consequences. Endometriosis has been proposed as one of the most commonly-encountered diseases of gynaecology, with the incidence of endometriosis in the general population being estimated to be around 5%, although it is thought that at least 20 25% of women in their thirties and forties may be suffering from this disease.

The VEGF family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and PLGF as well as their spliced variants. The biological activity of the VEGF family is mediated *in vivo* by three receptor tyrosine kinases that are primarily expressed in endothelial cells: kinase insert domain-containing receptor (KDR/FLK-1); the FMS-like 25 tyrosine kinase receptor-1 (FLT-1); and the FMS-like tyrosine kinase receptor-4 (FLT-4) which exhibit high binding affinity for the VEGF family members (de Vries *et al.*, (1992) *Science* 255: 989-991; Terman *et al.*, (1992) *Biochem. Biophys. Res. Commun.* 187: 1579-1586; Davis-Smyth *et al.*, (1996) *EMBO Journal* 15: 4919-4927). Additionally, the VEGF-A 165 isoform binds to neuropilin-1 (Soker S *et al.* (1998) *Cell* 92(6): 735-745) and 30 neuropilin-2 (Neufeld G *et al.* (1999) *FASEB J.*, 13(1): 9-22).

All of FLT-1, KDR/FLK-1 and FLT-4 are membrane-spanning receptors with an extracellular ligand-binding region containing seven immunoglobulin-like domains, a transmembrane domain and an intracellular tyrosine kinase domain. The transmembrane domain serves to anchor the receptor in the membrane of the cells in which it is expressed.

- 5 The biological activities of the FLT-1 and KDR receptors have been shown to differ, implying that these proteins have different functions *in vivo* (Roeckl *et al.*, (1998) Experimental Cell Research 241: 161-170; Shalaby *et al.*, (1995) Nature 376: 62-66; Fong G *et al.*, (1995) Nature 376: 66-70). FLT-4 is not a receptor for VEGF-A but rather binds to VEGF-C and VEGF-related protein (VRP). Like VEGF, VEGF-C and VRP can induce
10 mitogenesis in vascular endothelial cells, but at 100-fold less potency (Lee J *et al.*, (1996) Proc. Natl. Acad. Sci. USA 93: 1988-1992).

- Various groups have investigated the structure of VEGF receptors in relation to the biological functions of these molecules. Keyt *et al.*, (1996) (J. Biol. Chem. 271 (10) 5638-5646) mapped the residues important for VEGF binding on both KDR and FLT-1 and
15 suggested that VEGF displays different receptor binding sites for KDR and FLT-1.

- Shinkai *et al.*, (1998) (Journal of Biological Chemistry 273 (47): 31283-31288) mapped various sites of the extracellular domain of the KDR receptor thought to be involved in ligand association and disassociation and concluded that the third Ig-like domain is critical for ligand binding with the second and fourth domains playing a role in ligand association.
20 The fifth and sixth domains are required for retention of the ligand when bound to the receptor molecule, while the first Ig-like domain was proposed to regulate ligand binding.

- Davis-Smyth *et al.*, (1996) (EMBO Journal 15 (18): 4919-4927) reported that the second Ig-like domain of the FLT-1 receptor contains critical determinants of ligand binding. Furthermore, when the FLT-4 domain 2 was exchanged for that of FLT-1, FLT-4 became
25 non-responsive to its natural ligand VEGF-C, suggesting that domain 2 is also critical for binding in the FLT-4 receptor. These findings suggested that determinants for binding and ligand specificity within the second Ig-like domain is a common feature of subclass III receptor tyrosine kinases with seven Ig-like domains. Later studies proved that domains 2 and 3 are necessary for ligand binding with wild type affinity (Barleon *et al.*, (1997) J. Biol. Chem. 272 (16): 10382-10388; Davis-Smyth *et al.*, (1998) 273 (6): 3216-3222; Wiesmann *et al.*, (1997), Cell 91: 695-704). The ligand-FLT-1 domain 2 interactions were

determined in detail by the determination of the high resolution structure of FLT-1 domain 2 with VEGF (Wiesmann *et al.*, 1997, Cell 91; 695-704).

In an independent study, Barleon *et al.* (1997) (J. Biol. Chem. 272 (16); 10382-10388) mapped the sites for ligand binding and receptor dimerisation in the extracellular domain 5 of the FLT-1 receptor and confirmed that the first three Ig-like loops are involved in high affinity binding of VEGF. Dimerisation of the extracellular domains of FLT-1 receptor was only detected in the constructs that contain the fourth Ig-like loop.

- Kendall & Thomas, 1993 (P.N.A.S. USA 90; 10705-10709) reported the cloning of a soluble truncated form of FLT-1 from a human vascular endothelial cell library. This 10 molecule was found to comprise the six N terminal immunoglobulin-like extracellular ligand-binding domains but to lack the transmembrane-spanning region and intracellular tyrosine kinase domains. Binding affinity for VEGF-A was retained, prompting these workers to speculate that this soluble receptor might act as an efficient specific antagonist of VEGF *in vivo*.
- 15 Clark *et al.*, (1998) (Biol. Reprod. 59: 1540-1548) reported the occurrence of soluble FLT-1 (sFLT-1) in serum from pregnant women, which was not present in serum from men and from non-pregnant women. These workers thus speculated the *in vivo* production of the FLT-1 receptor might constitute a mechanism for naturally-regulating VEGF-induced angiogenesis. No naturally-occurring secreted form of KDR has been reported to date, 20 however, sFLT-1 has been shown to form ligand-induced heterodimeric complexes with full length KDR (Kendall *et al.*, (1996), Biochem. Biophys. Res. Commun. 226 (2): 324-328). To the best of the Applicant's, there is no naturally-occurring secreted form of FLT-4.

Certain approaches have been suggested that attempt to treat VEGF mediated disease by 25 supplying VEGF antagonists such as neutralising antibodies, VEGF receptor molecules, and portions of such receptor molecules (see, for example co-pending patent application PCT/GB95/01213, Metris Therapeutics; PCT/US97/17044, Merck & Co., Inc.; PCT/US97/07694, Genentech, Inc.; PCT/US92/09218, Genentech, Inc.; PCT/US94/01957, Merck & Co., Inc.). However, all of the approaches embodied in these patent applications 30 rely on reducing the effective concentration of VEGF molecules, and none of the suggested approaches have yet provided agents that are effective against all types of VEGF-mediated disease.

There thus remains a great need for novel compounds that are effective to disrupt VEGF function *in vivo*.

Summary of the Invention

According to a first aspect of the invention there is provided a protein consisting of the
5 amino acid sequence of the fourth Ig-like domain of a VEGF receptor, a variant of said protein that retains the ability to bind to a VEGF receptor or a functional equivalent of said fourth Ig-like domain.

All VEGF receptors form homodimers. The VEGF molecule itself acts as a dimer, and the binding of one monomer component to a receptor molecule induces the dimerisation of the
10 VEGF receptor molecule through the interaction of the second monomer component with a second VEGF receptor in the cell membrane (Fuh *et al.*, 1998, J. Biol. Chem. 273, No.18, 11197-11204). The dimerisation of the VEGF receptor molecule induces the activation of the intracellular kinase domain of the receptor, thus initiating the signal transduction cascade that is effective to translate the ligand-receptor binding event into the activation of
15 the appropriate secondary messenger system in the cell.

The present invention provides molecules that when bound to the fourth Ig-like domain of a full length VEGF receptor, prevent its dimerisation. Since VEGF-dependent activation of the intracellular signalling domain of the full-length receptor occurs only when the VEGF receptor is in its dimeric state, blocking the dimerisation event severs the link between
20 ligand binding and activation of the secondary messenger system. Accordingly, the biological action of VEGF may be specifically blocked.

The invention has a number of advantages over systems that have been previously described. Most of these systems involve mechanisms that are designed to remove the effective amount of VEGF from circulation, either systemically, or in specific areas of the
25 body. Such techniques are far from ideal for a number of reasons, the most obvious being that VEGF is a molecule with a wide range of biological functions in the body. Lowering the effective levels of this molecule, either by preventing its expression, or by interfering with it directly through specific binding events, acts to abolish VEGF function altogether, so leading to unwanted side-effects. Studies that have used small molecule inhibitors to
30 target VEGF-Receptor tyrosine kinases may inhibit other kinases and so cause unwanted side-effects.

One advantage of using the molecules of the present invention in therapy is that VEGF itself is left unaffected, so a free population of the VEGF molecule remains to perform its natural biological functions. By targeting the receptor molecules themselves, VEGF levels remain unchanged, meaning that the normal VEGF-mediated processes are allowed to 5 continue unaltered by the therapy process. Furthermore, the molecules of the present invention may be designed to target only a subset of VEGF receptor types, so leaving non-targeted receptors unaffected, meaning that this method of therapy is unlikely to cause undesirable side-effects.

By the term the "fourth Ig-like domain" is meant the immunoglobulin-like domain of the 10 VEGF receptor that is considered by the inventors to be necessary for the dimerisation function of the receptor molecule. This domain is defined as being the fourth Ig-like domain as counted from the NH₂ terminus of the receptor molecule. It is not at present clear which precise residues participate in the dimerisation event. However, the molecules of the invention should retain sufficient residues from the fourth Ig-like domain to bind to 15 the corresponding domain of a VEGF receptor with high enough affinity to compete effectively for binding with wild type full length VEGF receptor molecules.

At present, there are three VEGF receptors known. However, the present invention is likely to be equally applicable to other VEGF receptors that are discovered in the future. Preferably, the proteins of the present invention are derived from the receptors FLT-1, 20 FLK/KDR and FLT-4. The VEGF receptors to which the proteins of the invention bind are preferably mammalian, most preferably human VEGF receptors.

In FLT-1, the boundaries of the fourth Ig-like domain are considered to be within amino acid residues 316 or 317 and 447 inclusive, wherein the numbering system starts at the first methionine residue in Figure 1. However, shorter protein molecules may be used, provided 25 that the molecules include at least amino acid residues 344-406 of the FLT-1 sequence. Preferably, those proteins of the invention that are derived from FLT-1 consist of at least residues 338-440, more preferably 330-440 of the full length FLT-1 sequence. Examples of particularly preferred constructs include those that consist of residues 330-440, 330-429 or 338-429 of the full length FLT-1 sequence.

30 The analogous residues in the FLK receptor are shown in Figure 5. The boundaries of the fourth Ig-like domain of this receptor is considered to be at residues 314 and 440 of the full length FLK sequence given in Figure 2. At the very least, this domain should include

residues 342-404 of the FLK sequence. Preferably, those proteins of the invention that are derived from FLK consist of at residues 336-439, 335-435, 335-424, more preferably residues 328-424 or 328-435 of the full length FLK sequence.

- The analogous residues in the KDR receptor are also shown in Figure 5. The boundaries of
- 5 the fourth Ig-like domain of this receptor are considered to be at residues 312-438 of the full length sequence given in Figure 3. At the very least, this domain should include residues 340 to 402 of the KDR sequence. Preferably, those proteins of the invention that are derived from KDR consist of at residues 333-438, 333-433 or 333-422, more preferably residues 326-422 or 326-433 of the full length KDR sequence.
- 10 The analogous residues in the FLT-4 receptor are also shown in Figure 5. The boundaries of the fourth Ig-like domain of this receptor are considered to be residues 315 to 437 of the full length sequence given in Figure 4. At the very least, this domain should include residues 343-403 of the FLT-4 sequence. Preferably, those proteins of the invention that are derived from FLT-4 consist of residues 339-437, 339-423, more preferably, 329-423 or
- 15 329-437 of the full length FLT-4 sequence.

The boundaries of the fourth Ig-like domain were predicted, based on sequence alignments of FLT-1 and KDR with telokin, as well as secondary structure predictions assisted by the crystal structure of the telokin molecule (PDB file: 1TLK).

- The maximum boundaries of the fourth Ig-like domain were determined to reside within a
- 20 few residues of the last conserved cysteine residue in the third Ig-like domain, and the first conserved cysteine residue of the fifth Ig-like domain (Cys311 and Cys454 respectively).

The minimum boundaries were defined after aligning FLT-1 to telokin and defining the minimum number of secondary structure elements that would be sufficient to support a stable protein fold.

- 25 Additionally, information of the exon-intron genomic organisation of FLT-1 was generated by the Applicant, constructed from sequencing data generated from the human genome sequencing effort. FLT-1 resides within chromosome 13; the genomic organisation of this gene was deciphered using recently deposited sequencing data of chromosome 13 clones.

- 30 The identification of the appropriate boundaries of other VEGF receptors to which the teaching of the present invention may be applied will be clear to those of skill in the art. Details of the boundaries of other preferred constructs are shown in Table 1.

The proteins of the present invention may comprise an amino acid sequence that corresponds exactly to the wild type receptor protein sequence found in the fourth Ig-like domain of the VEGF receptor protein. The wild type amino acid sequences of the fourth Ig-like domains of the FLT-1, FLK/KDR and FLT-4 receptors are shown in Figure 5.

- 5 However, as the skilled reader will appreciate, the proteins of the invention may be derived from any mammalian VEGF receptor sequence. Human sequences are preferred.

As used herein, the term "wild type" means the amino acid sequence that is characteristic of most of the members of the particular species from which the receptor molecule is derived. Included within the term "wild type" are natural biological variants of the VEGF
10 receptor molecule sequences (for example, allelic variants or geographical variations within the species from which the wild type proteins are derived).

The proteins of the present invention may most suitably be derived from VEGF receptors in the FLT-1, KDR/FLK and FLT-4 receptor family. All of these molecules dimerise through the interaction of the respective fourth Ig-like domains of the molecule. Of
15 particular applicability to the present invention are recombinant proteins derived from the FLT-1 and KDR/FLK proteins. These molecules, and the effect of these molecules when aberrantly-regulated or when mutated, is thought to have a particularly important role in the pathology of diseases such as cancer and endometriosis.

Variants of wild type fourth Ig-like domain receptor sequences are also included in the
20 present invention. As the skilled man will appreciate, the term "variant" includes molecules that contain single or multiple amino-acid substitution(s), addition(s), insertion(s) and/or deletion(s) from the wild type protein sequence, provided that such variants maintain the ability to bind to the corresponding fourth Ig-like domain of target VEGF receptor and thus prevent their dimerisation. Variant molecules may also contain substitutions of chemically-
25 modified or synthetic amino acids that do not affect the function or activity of the protein in an adverse manner.

Suitable variants of the molecules of the invention will be those proteins that exhibit high affinity for the fourth Ig-like domain of a VEGF receptor. Preferably, this affinity is higher than that of the wild type sequence. Typically, the protein according to the present invention
30 binds to a VEGF receptor with a dissociation constant of $2\mu\text{M}$ or less, preferably, $0.2\mu\text{M}$ or less, more preferably 2nM or less, even more preferably, 20pm or less.

- Another property that is desirable for a variant of the wild type sequence is the ability to bind to the fourth Ig-like domain of an intact VEGF receptor with a significantly higher affinity than the wild type protein displays for binding to the intact VEGF receptor. The dimerisation kinetics will lead towards the formation of heterodimers with target receptor with increasing
- 5 concentrations of the proteins of the invention. Such concentrations will vary for different cell types as they will depend on the number of target receptor molecules that are present on the cell surface. However, a large excess of protein should be able to mask all cell surface receptors, so conferring a therapeutic effect to the patients to whom preparations of these proteins will be administered.
- 10 The term "functional equivalent" is used herein to describe proteins that have an analogous function to the fourth Ig-like domain of a VEGF receptor and that bind specifically to this domain, thus preventing dimerisation of the receptor molecules and so inhibiting signal transduction effected by ligand binding to the VEGF receptor. This term therefore includes molecules that are structurally similar to the fourth Ig-like domains identified herein or that
- 15 contain a similar or identical tertiary structure. The analogous binding properties of functional equivalents should be reflected in their affinity for the fourth Ig-like domain of a VEGF receptor. Typically, functional equivalents should bind to a VEGF receptor with a dissociation constant of $2\mu\text{M}$ or less, preferably, $0.2\mu\text{M}$ or less, more preferably 2nM or less, even more preferably, 20pm or less. For these molecules, the thermodynamics of
- 20 binding should be sufficient that physiologically-attainable concentrations of molecule are effective to prevent VEGF receptor dimerisation.
- The term "functional equivalent" therefore includes entities such as antibodies, (particularly antiidiotypic antibodies), oligopeptides, peptides, peptidomimetics, drug molecules such as small natural or synthetic organic molecules of up to 2000Da, preferably
- 25 800Da or less in size. Other examples of functional equivalent molecules will be clear to those of skill in the art.
- Functionally-equivalent peptide, oligopeptide or polypeptide compounds according to the present invention may be generated by any suitable means, as will be apparent to those of skill in the art. In addition to the naturally-occurring amino acids, these molecules may, of
- 30 course, contain synthetic amino acids.

In the case of antiidiotypic antibodies, these may be obtained by immunisation of an appropriate host with a preparation of an antibody that recognises the dimerisation interface within the fourth Ig-like domain molecule.

In the case of peptides, combinatorial peptide libraries may be most suitable to isolate
5 peptide molecules that display the desired binding characteristics, through the use of selection regimes that select for molecules that bind to antibodies that are specific for the fourth Ig-like domain of a VEGF receptor.

One method of generation of peptide libraries utilises degenerate oligonucleotide libraries. This method allows the subsequent analysis of the encoding nucleic acid and thus gives
10 direct sequence information for the mimotope (see for example, Cull *et al.* (1992); Matteakis *et al.*, (1994)).

Phage display technology also provides a vehicle that allows for the selection of displayed peptides, oligopeptides or polypeptides and that simultaneously provides a link between phenotype and genotype so that the encoding nucleic acid can be identified and analysed
15 (for a review see Clackson and Wells (1994) Trends Biotechnol 12: 173-184). Filamentous phage particles act as genetic display packages with proteins on the outside and the nucleic acids that encode them on the inside. The practical limit on library size allowed by this technology is of the order of 10^7 to 10^{11} variants, so allowing the generation of a huge number of different compounds. This technology also allows iterative rounds of selection
20 to be performed, so honing the affinity of the molecules isolated.

The preferred method of generation of peptide, oligopeptide or polypeptide compounds that are functional equivalents of the proteins of the invention is through selection of candidate compounds in a phage display library.

Selection of a nucleic acid or gene from a phage display library will in most cases require
25 the screening of a large number of variant nucleic acids or genes. Libraries of nucleic acids or genes for use with phage display technology may be generated in a variety of ways. For example, pools of naturally-occurring genes may be cloned from genomic DNA or cDNA (see Sambrook *et al.*, 1989). Phage-antibody libraries, made by PCR amplification repertoires of antibody genes from immunised or non-immunised donors have proved very
30 effective sources of functional antibody fragments (Winter *et al.*, (1994) Annu Rev Immunol, 12: 433-55; Hoogenboom, (1997) Trends Biotechnol. 15: 62-70).

Libraries of genes can also be made by encoding all or part of genes or pools of genes or by using randomised or doped synthetic oligonucleotides. Libraries can also be made by randomly introducing mutations into a gene or into a pool of genes by a variety of techniques *in vivo*, including using so-called 'mutator strains' of bacteria such as *E. coli mutD5* (Liao *et al.*, (1986) *P.N.A.S.U.S.A.*, **83**: 576-580).

Random mutations can also be introduced both *in vivo* and *in vitro* by chemical mutagens, and ionizing or UV irradiation (see Friedberg *et al.*, (1995) *DNA repair and mutagenesis*. ASM Press, Washington), or by incorporation of mutagenic base analogues (Zaccolo *et al.*, (1996) *J Mol Biol* **255**: 589-603). Mutations can also be introduced into genes *in vitro* during 10 polymerisation, for example by using error-prone polymerases (Leung *et al.*, (1989) *Technique*, **1**: 11-15). Further diversification can be introduced by using homologous recombination either *in vivo* (see Kowalczykowski *et al.*, (1994) *Microbiol Rev*, **58**: 401-465) or *in vitro* (Stemmer, (1994) *Nature*, **370**: 389-391). Alternatively, directed mutagenesis may be performed according to methods well known in the art (see McPherson *et al.*, (1991) 15 *Directed mutagenesis. A Practical Approach*. IRL Press, Oxford).

The proteins may be specific for a class of VEGF receptors for example, the proteins may bind with high affinity to both the FLT-1, KDR/FLK and FLT-4 VEGF receptors, thus abolishing VEGF-mediated activity via all these receptor types.

In an alternative embodiment, the proteins of the invention may be specific for one 20 particular type of receptor, for example, FLT-1. In this manner, specific VEGF pathways can be targeted, that are responsible for a particular disease, or for a particular aspect of a disease. In this manner, the risk of toxic side-effects can be reduced.

In a further embodiment, the proteins of the invention may comprise hybrid molecules consisting of multiple components. Such proteins of the invention may consist of repeated 25 amino acid sequences of the fourth Ig-like domain of one or more VEGF receptors. Proteins of this aspect of the invention may dimerise more efficiently to VEGF receptor target molecules through possessing multiple binding sites.

In an alternative embodiment, such repeated domains may be derived from different receptors. For example, a protein may comprise an amino acid sequence derived partially 30 from the fourth Ig-like domain of the FLT-1 receptor and partially from the fourth Ig-like domain of the KDR/FLK or FLT-4 receptor. In this manner, proteins according to the

invention may be designed rationally so as to impart specific binding properties of interest, such as increased affinity for a certain receptor molecule. The protein may thus be designed so as to interfere with a specific dimerisation event, for example, the dimerisation of the FLT-1 receptor. The normal dimerisation process exhibited by other VEGF receptors, for example a KDR receptor, may therefore be unaffected.

- According to a further aspect of the invention there is provided a protein or functional equivalent according to any one of the above-described aspects of the invention that has been genetically or chemically fused to one or more peptides or polypeptides. For example, dimerisation of the proteins of the invention with each other might be prevented by fusing
- 10 the fourth Ig-like domain to an effector domain that acts in the absence of ligand to prevent homodimerisation of the protein. On binding of a ligand to the effector domain, a change in the conformation of the fusion protein would allow dissociation of the fourth Ig-like domain portion of the protein, leaving it free to bind to its target VEGF receptor molecule *in situ*.
- 15 Other components suitable for fusion with proteins of the invention include labels, such as a radioactive, enzymatic, fluorescent, or antibody label. In this embodiment of the invention, fusion proteins can be used as diagnostic tools in the evaluation of the disease state of a patient. Other suitable components for fusion include bioactive moieties such as toxins that could be delivered to specific cell types.
- 20 The proteins of the invention are preferably recombinant, meaning that they are derived by recombinant DNA technology. Recombinant expression of proteins allows a high level of expression to be obtained at an economic cost. Recombinant expression is widely known in the art and involves the incorporation of the gene encoding the protein of interest into an expression vector. Such an expression vector will incorporate appropriate transcriptional
- 25 and translational control sequences, for example enhancer elements, promoter-operator regions, termination stop sequences, mRNA stability sequences, start and stop codons or ribosomal binding sites, linked in-frame with the gene encoding the protein of interest. Secretion signalling and processing sequences may also be appropriate. Many suitable vectors and expression systems are well known and documented in the art (see, for
- 30 example, Sambrook *et al.*, Molecular Cloning: a laboratory manual; Cold Spring Harbor Laboratory Press; Fernandez & Hoeffler, 1998). Particularly suitable viral vectors include baculovirus-, adenovirus- and vaccinia virus-based vectors.

The proteins of the invention may be expressed recombinantly in prokaryotic hosts, such as in *E. coli*, or in eukaryotic yeasts that can be made to express high levels of recombinant protein and that can be grown easily in large quantities. Mammalian cell lines grown *in vitro* are also suitable, particularly when using virus-driven expression systems. Another 5 suitable expression system is the baculovirus expression system that involves the use of insect cells as hosts. An expression system may also constitute host cells that have the encoding DNA incorporated into their genome. Recombinant protein may easily be purified from these host cells in large quantities and at an economic cost.

Proteins for the treatment of diseases such as cancer or endometriosis will generally be 10 administered to patients as pharmaceutical compositions in therapeutically-effective doses. The term "therapeutically effective dose" as used herein refers to an amount of a therapeutic agent that is effective to treat, ameliorate, or prevent the disease in question, or to exhibit a detectable therapeutic or preventative effect. The precise effective amount for a subject will depend upon the subject's size and health, the nature and extent of the disease 15 condition, and the therapeutic agent or combination of therapeutic agents selected for administration. The effective dose for each given situation can be determined by routine experimentation and is within the judgement of the skilled person. For example, in order to formulate a range of dosage values, cell culture assays and animal studies can be used.

The dosage of such compounds preferably lies within the dose that is therapeutically 20 effective, and that exhibits little or no toxicity at this level. For example, an effective parenteral dose will be between 0.01 mg/ kg and 50 mg/kg or, more typically, between 0.05 mg/kg and 10 mg/kg of the individual to which it is administered.

According to a further aspect of the invention there is provided a protein according to any one of the above-described aspects of the invention, for use as a pharmaceutical.

- 25 The invention also provides the use of a protein according to any one of the above-described aspects of the invention in the manufacture of a medicament for the treatment of cancer, endometriosis, inflammation, psoriasis, rheumatoid arthritis, hemangiomas, diabetic retinopathy, angiofibromas, macular degeneration, retinal neovascularisation or any other disorder whose pathology is dependent upon a VEGF family-mediated pathway.
- 30 Specific neoplasms and neoplastic conditions that are amenable to treatment include breast carcinomas, lung carcinomas, gastric carcinomas, oesophageal carcinomas, colorectal

carcinomas, liver carcinomas, ovarian carcinomas, thecomas, arrhenoblastomas, cervical carcinomas, endometrial carcinomas, endometrial hyperplasias, fibrosarcomas, choriocarcinomas, head and neck cancers, nasopharyngeal carcinomas, hemangiomas, laryngeal carcinomas, hepatoblastomas, Kaposi's sarcomas, melanomas, skin carcinomas,
5 cavernous hemangiomas, hemangioblastomas, pancreas carcinomas, retinoblastomas, astrocytomas, glioblastomas, Schwannomas, oligodendrogiomas, medulloblastomas, neurblastomas, rhabdomyosarcomas, osteogenic sarcomas, leiomyosarcomas, urinary tract carcinomas, thyroid carcinomas, Wilm's tumour, renal cell carcinomas, prostate carcinomas, abnormal vascular proliferation associated with phakomatoses, oedemas (such
10 as that associated with brain tumours) and Meig's syndrome.

Specific non-neoplastic conditions that are amenable to treatment include rheumatoid arthritis, psoriasis, atherosclerosis, diabetic and other retinopathies, retrobulbar fibroplasias, leiomyomas, neovascular glaucomas, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, chronic inflammation, lung inflammation,
15 nephrotic syndrome, pre-eclampsia, ascites, pericardial effusion (such as that associated with pericarditis) and pleural effusion.

The binding of proteins of the invention, or functional equivalents thereof, to the full length VEGF receptor or to truncated forms thereof can be used in high throughput screens for the identification of small molecular weight drug substances, such as small natural or
20 synthetic organic molecules of up to 2000Da, preferably 800Da or less in size that are effective in inhibiting dimerisation of a VEGF receptor. These compounds may be peptidic or non-peptidic in nature. The assays can be radioactive, fluorescent, colorimetric, enzymatic, chemiluminescent or any other assay type that allows the quantitation of bound substance. In an alternative to the use of binding assays, enzyme assays such as tyrosine
25 kinase assays may be used to assess the degree to which signal transduction is inhibited.

According to a still further aspect of the invention there is provided a nucleic acid encoding a protein according to any one of the above-described aspects of the invention. Such nucleic acid molecules may be incorporated into a suitable vector, which itself may be used to transfet a suitable host cell.

30 Nucleic acid molecules according to this aspect of the invention may in one aspect be used in the recombinant expression of the proteins of the above-described aspects of the invention.

In another aspect, such nucleic acid molecules may be used in gene therapy, to effect expression of the protein *in situ*. Gene therapy vehicles may comprise non-viral agents such as liposome formulations or may comprise recombinant viral vectors. Suitable viral vectors include, for example, vectors derived from retroviruses, adenoviruses, adeno-
5 associated viruses, herpes viruses or papilloma viruses. Non-viral vectors include simple plasmids formulated, for example, as liposomes (Templeton and Lasic, (1999) Mol Biotechnol 11(2):175-80). Expression of the coding sequence can be induced using endogenous mammalian or heterologous promoters, and may be either constitutive or regulated. Suitable techniques for the introduction of gene therapy vehicles into cells
10 include electroporation, the use of DNA guns, the direct injection of pure nucleic acid into tissue and liposome-mediated techniques (Dachs *et al.*, (1997) Oncol. Res. 9(6-7): 313-325; Templeton and Lasic, (1999) Mol Biotechnol 11(2): 175-80). Gene therapy vehicles may be administered either locally or systemically.

An alternative form of gene therapy involves the introduction of cells, preferably
15 autologous host cells, that contain nucleic acid sequences according to the above-described aspects of the invention into a patient suffering from a VEGF-mediated disease or disorder. The cells may comprise autologous cells harvested from the patient and transfected *ex vivo* with one or more nucleic acids encoding proteins according to the above-described aspects of the invention. These cells may then be transplanted back into the patient in areas that
20 allow for the amelioration of disease symptoms to restore the healthy function of the tissue and prevent disease progression (Bailey C.J. *et al.*, (1999) J. Mol. Med. 77(1): 244-249; Falqui L. *et al.*, (1999) Hum. Gene. Ther. 10(11): 1753-1762).

According to a further aspect of the invention there is provided a pharmaceutical composition comprising a protein or a nucleic acid according to any of the above-described
25 aspects of the invention, in conjunction with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" includes large, slowly metabolised macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers and inactive virus particles. Pharmaceutically acceptable salts may also be used, for example, mineral acid salts such as
30 hydrochlorides, hydrobromides, phosphates, and sulphates, or salts of organic acids such as acetates, propionates, malonates, benzoates (see Remington's Pharmaceutical Sciences (Mack Pub. Co., N.J. 1991)). Pharmaceutically acceptable carriers in therapeutic

compositions may also contain liquids such as water, saline, glycerol and ethanol. Additionally, auxiliary substances, such as wetting or emulsifying agents and pH buffering substances, may be present.

Typically, pharmaceutical compositions are prepared as injectables, either as liquid
5 solutions or suspensions, or for application in patches. Solid forms suitable for solution in,
or suspension in liquid vehicles prior to injection may also be prepared. Preparations for
oral administration may also be formulated to allow for controlled release of the active
compound. For administration by inhalation, the compounds of the invention may be
delivered by any means known to those of the skill in the art, such as using aerosol sprays,
10 capsules or cartridges.

According to a further aspect of the invention there is provided a method for treating a patient suffering from a disorder whose pathology is dependent upon a VEGF-mediated metabolic pathway comprising administering to a patient a therapeutically-effective amount of a protein, a nucleic acid encoding such a protein, or a pharmaceutical
15 composition according to any one of the above-described aspects of the invention.

According to a still further aspect of the invention there is provided a genetically-modified animal, such as a transgenic animal, particularly a transgenic rodent animal, which expresses a protein according to any one of the above-described aspects of the invention. Methods for the production of genetically-modified animals are known in the art and
20 include techniques such as modification of somatic cells, or germ line therapy to incorporate heritable modifications (see, for example, Rajewsky *et al.*, (1996), J Clin Invest 98, 600-3; Metzger and Feil, (1999) Curr. Opinions Biotechnology 10, 470-476; Bedell *et al.* (1997), Genes Dev. 11: 11-43; Bedell *et al* (1997), Genes Dev. 11: 1-10; "Transgenic Mammals", John Bishop (1999) Pearson Education Ltd., Harlow, Essex, for
25 example, p228). Preferably, transgenic organisms are created using germ line gene therapy.

According to a still further embodiment of the invention, there is provided a method for inhibiting the dimerisation of a VEGF receptor, comprising bringing the receptor into contact with a protein, or functional equivalent according to any one of the embodiments of the invention described above.

Various aspects and embodiments of the present invention will now be described in more detail by way of example. It will be appreciated that modification of detail may be made without departing from the scope of the invention.

BRIEF DESCRIPTION OF THE FIGURES

- 5 Figure 1 gives the sequence of the *Homo sapiens* FLT-1 gene (acc. no. NM_002019).
- Figure 2 gives the sequence of the *Mus musculus* FLK-1 gene (acc. no. X70842).
- Figure 3 gives the sequence of the *Homo sapiens* KDR/flk-1 gene (acc. no. AF035121).
- Figure 4 gives the sequence of the *Mus musculus* Flt-4 gene (acc. no. NM_008029).
- Figure 5 shows an alignment of the most relevant parts of the fourth Ig-like domains of the
- 10 FLT-1, FLK, KDR and FLT-4 protein sequences. Residues 316-447 of domain IV of
FLT-1 are shown aligned to residues 312-438 of KDR, 314-440 of FLK and 315-437 of
FLT-4.
- Figure 6 presents a Coomassie-stained SDS-PAGE gel showing the purification of His-Tagged Domain 4 (Construct 0) from *E. coli* crude soluble extract. Lane 1 shows unbound
- 15 crude protein from His-tag column after incubation with 2M Urea buffer; Lane 2 shows the bound protein after elution with 400 mM imidazole; Lane 3 shows unbound crude protein from His-tag column after incubation with 4 M Urea buffer; Lane 4 shows the bound protein after elution with 400 mM imidazole; Lane 5 shows unbound crude protein from the His-tag column after incubation with 6 M Urea buffer; and Lane 6 shows the bound
- 20 protein after elution with 400 mM imidazole.
- Figure 7 presents a Coomassie-stained SDS-PAGE gel showing all four constructs of Domain 4 after His-tag purification. Lanes 1 to 4 show the step-wise purification of construct 0 (14.16 kDa) with increasing concentrations of imidazole to a final elution concentration of 400 mM imidazole. Lane 1 shows unbound crude protein from construct
- 25 0; lane 2 shows eluant after 50 mM imidazole wash; lane 3 shows eluant after 100 mM imidazole wash; and lane 4 shows eluant after 400mM imidazole wash. Lanes 5 and 6 show purification of construct 1 (13.8 kDa) after a final wash step with 100 mM imidazole (lane 5) and elution with 400 mM imidazole (lane 6). Lanes 7 and 8 show purification of construct 2 (15.12 kDa) after a final wash step with 100 mM imidazole (lane 7) and elution
- 30 with 400 mM imidazole (lane 8) and finally lanes 9 and 10 show purification of construct 3

(12.84 kDa) after a final wash step with 100 mM imidazole (lane 9) and elution with 400 mM imidazole (lane 10).

Figure 8 shows the specific detection of the four Domain 4 constructs by Western blotting using an anti-sFLT-1 polyclonal antibody. Lanes 1 and 2 show construct 0, lanes 3 and 4 construct 1, lanes 5 and 6 construct 2 and lanes 7 and 8 construct 3.

Figure 9 shows the specific detection of the four Domain 4 constructs by Western blotting using an anti-penta Histidine monoclonal antibody. Lane 1 shows construct 0, lane 2 construct 1, lane 3 construct 2 and lane 4 construct 3.

Figure 10 presents BIACORE data of the binding of sFLT-1 in the presence and absence of all four Domain 4 constructs.

Figure 11 presents BIACORE data of the binding of all four Domain 4 constructs on the chip.

Figure 12 shows slot blot data of Domain 4 constructs 0, 1 and 2 screened with hybridomas 1H11, 6C5, 18G12, and 15F8.

Table 1 shows the boundaries of preferred constructs for the FLT-1, KDR, FLK and FLT-4 VEGF receptors.

Table 2 demonstrates the percentage of inhibition achieved by all four Domain 4 constructs on the binding of the sFLT-1 molecule on to the 2B2 antibody

EXAMPLES**Example 1: Preparation of FLT-1 Domain 4 molecules****Construct 0**

338 HRKQQVLETVAGKRSYRLSMKVKAFPSPEVVWLKDGLPATEKSARYLTRGYSL

5 IIKDVTEEDAGNYTILLSIKQSNVFKNLTATLIVNVKPQIYEKAVSSFPD 440

Construct 1

330 DKA~~FITVKHR~~KQQVLETVAGKRSYRLSMKVKAFPSPEVVWLKDGLPATEK

SARYLTRGYSLIKDVTEEDAGNYTILLSIKQSNVFKNLTATLIVNVKPQ 429

Construct 2

10 330 DKA~~FITVKHR~~KQQVLETVAGKRSYRLSMKVKAFPSPEVVWLKDGLPATEK

SARYLTRGYSLIKDVTEEDAGNYTILLSIKQSNVFKNLTATLIVNVKPQIYEKAV

SSFPD 440

Construct 3

338 HRKQQVLETVAGKRSYRLSMKVKAFPSPEVVWLKDGLPATEKSARYLTRG

15 YSLIKDVTEEDAGNYTILLSIKQSNVFKNLTATLIVNVKPQ 429

The protein sequences of four potential Domain 4 constructs of FLT-1 are given above and contain residues 338-440 (Construct 0), residues 330-429 (Construct 1), 330-440 (Construct 2) and 338-429 (Construct 3) respectively. The underlined residues are predicted to be strands by homology alignments to the known structures of telokin (1TLK) and domain 2 of FLT-1 (1FLT).

Any of the above constructs of the fourth Ig-like domain may be cloned by polymerase chain reaction (PCR) from the full length sFLT-1 clone using the upstream primer

Construct 0

25 (D4for) Sense 5' GGGAATTCCATATGCATCGAAAACAGCAGGTGCTTGAAAC 3',
containing an *Nde*I site and the downstream primer
(D4rev)Antisense 5' CGCGGATCCTTAGTCTGGAAACGATGACACGGC 3'

containing an artificial stop codon and a BamHI restriction site.

Construct 1

(Flt 39) Sense 5'CCGGTATCCATATGGATAAAGCATTCACTCACTGTG3'

containing an NdeI site and the downstream primer

5 (Flt 40) Antisense 5'CGCGGATCCTACTGGGGTTTCACATTGACAATTAGAG 3'

containing an artificial stop codon and a BamHI restriction site.

Construct 2

(Flt 39) Sense 5'CCGGTATCCATATGGATAAAGCATTCACTCACTGTG3'

containing an NdeI site and the downstream primer

10 (D4rev) Antisense 5'CGCGGATCCTAGTCTGGAAACGATGACACGGC3'

containing an artificial stop codon and BamHI restriction site.

Construct 3

(D4for) Sense 5'GGGAATTCCATATGCATCGAAAACAGCAGGTGCTTGAAAC3'

containing an NdeI site and the downstream primer

15 (Flt 40) Antisense 5'CGCGGATCCTACTGGGGTTTCACATTGACAATTAGAG 3'

containing an artificial stop codon and a BamHI restriction site. The regions complementary to the FLT-1 molecule are shown in bold.

PCR products are purified with the QIAquick gel extraction kit (QIAGEN) and digested with BamHI and NdeI. The digested fragments are purified with the QIAquick PCR

20 purification kit (Qiagen) and ligated into the *E.coli* vector pEE14b (Novagen) that was digested by NdeI and BamHI, followed by dephosphorylation using calf intestinal alkaline phosphatase. The presence of the insert was verified by double digestion with NdeI and BamHI.

For expression, the plasmid construct is transformed in *E. coli* strain BL21(DE3) carrying
25 the inducible T7 polymerase gene. Bacterial cultures of 1lt of LB medium containing 200 μ g/ml ampicillin are grown in shaking flasks at 37°C up to an optical density of 0.7 at 600nm. The culture is grown for another 4h after addition of 50 μ M isopropyl- β -D-thiogalactosidase at 25°C. Cells are harvested and the pellets frozen at -80°C.

- Cells are lysed by mild sonication in 50mM Tris-HCl, 300mM NaCl, 10% glycerol, 0.25mM PMSF pH 8.0. Any insoluble material is removed by centrifugation at 20000g for 30min at 4°C and the histidine-tagged protein is batch absorbed onto Ni-NTA-agarose (Qiagen). The resin is washed twice with sonication buffer, followed by two washes with
- 5 50mM Tris-HCl, 300mM NaCl, 5mM imidazole, 20% glycerol pH8.0. The protein is eluted from the resin with 50mM Tris-HCl, 100mM NaCl, 10% glycerol, 300mM imidazole pH8.0.
- Removal of the His-Tag is performed using 6 units of thrombin at 4°C overnight. The untagged protein is subsequently purified from other minor contaminants on a Superdex S-
- 10 100 gel-filtration column equilibrated in 20mM Tris-HCl, 150mM NaCl, pH8.0. The identity of the material is confirmed by N-terminal sequencing.

Alternative method for expression of FLT-1 Domain 4 molecules

- For expression, the plasmid construct was transformed in *E. coli* strain BL21(DE3)pLysS carrying the inducible T7 RNA polymerase gene and the cells were plated onto LB agar
- 15 containing 50µg/ml ampicillin, 34 µg/ml chloramphenicol and 1% glucose and incubated overnight at 37°C. A single colony of these cells are aseptically picked from the agar plate and inoculated into 5 ml LB broth containing 50 µg/ml ampicillin, 34 µg/ml chloramphenicol and 1 % glucose and grown with shaking (220 rpm) overnight at 37 °C. The following morning, 1 ml of the overnight suspension is used to inoculate fresh 100 ml
- 20 LB broth containing 50 µg/ml ampicillin, 34 µg/ml chloramphenicol and 1 % glucose and grown with shaking at 37 °C until an optical density of 0.7 at 600 nm is reached. The cells are then pelleted by centrifugation at 3500 rpm for 15 minutes at 4 °C and resuspended in fresh 100 ml LB media containing 50 µg/ml ampicillin, 34 µg/ml chloramphenicol 1 % glucose and 0.1 mM isopropyl-β-D-thiogalactosidase in order to induce protein expression.
- 25 Recombinant protein is produced from the cells for 16 hours at 16 °C. The cells are harvested and the pellets frozen at - 80 °C.

Cells lysis was performed by using Novagen's Bug Buster™ Protein Extraction Reagent following the manufacturer's instructions and both the soluble clarified protein as well as inclusion bodies are collected.

- 30 The crude soluble fraction containing the histidine-tagged Domain 4 variants was purified using an Ni-NTA agarose column (QIAGEN). Purification was performed under

denaturing conditions, since the His-Tag tends to interact with the protein backbone, so making the protein inaccessible to the resin. Batch purification was performed by incubating the crude material overnight in buffer containing 6 M Urea, 10 mM Tris-HCl, 100 mM NaH₂PO₄ pH 8.0 with pre-equilibrated Ni-NTA resin at room temperature. The 5 resin was removed by centrifugation and the unbound supernatant removed. The Ni-NTA agarose containing absorbed His-Tagged Domain 4 protein was resuspended in wash buffer containing 6M Urea, 10 mM Tris-HCl, 100 mM NaH₂PO₄, pH8.0 and loaded onto a column fitted with a 3μm filter. The column containing resin was step-wise washed with wash buffer containing increasing concentrations of imidazole (from 25 to 100 mM) and 10 the fusion protein eluted from the resin with wash buffer containing 400 mM imidazole, pH 8.0. The identity of the eluted Domain 4 fusion proteins was confirmed by SDS-Coomassie staining and Western blotting using both an anti-His_Tag monoclonal antibody as well as an anti-Flt polyclonal antibody.

Example 2: Preliminary Analysis of Domain 4 variants

15 • **SDS Page analysis of the four Domain 4 constructs**

15 μl aliquots of protein samples were added to 3 μl of 4 x NuPAGE LDS loading buffer (Novex) and 2 μl of 10 x NuPAGE sample reducing agent (Novex), and heated at 99 °C for 5 minutes prior to loading on a 4-12 % Bis-Tris SDS PAGE gel in NuPAGE MES SDS running buffer (Novex). Protein separation was carried out for 45 minutes at 200 V. 20 Proteins were stained with Coomassie blue stain for 45 minutes and destained for 1 hour with 10 % glacial acetic acid and 30 % methanol. The results are shown in Figures 6 and 7.

• **Western blotting analysis of the four Domain 4 constructs**

Western blotting of all four constructs was performed by using anti-sFLT-1 specific 25 antibody as follows;

After electrophoresis, gels were transferred to polyvinylidene difluoride (PVDF) membrane in NuPAGE transfer buffer (Novex) with antioxidant and 20 % methanol. The transferred membranes were blocked in phosphate buffered saline (PBS) with 1 % BSA for 1 hour at room temperature prior to incubation for 1 hour in PBS with 1 % bovine gamma 30 globulin, 0.05 % Tween-20 and polyclonal goat anti-sFLT-1 biotinylated antibody (0.2ug/ml). The membranes were then washed for 5 minutes in PBS and three times in

- PBS- 0.05 % Tween for 5 minutes, and finally washed for another 5 minutes in PBS. Thereafter the membranes were incubated in PBS with 1 % bovine gamma globulin, 0.05 % Tween-20 and polyclonal goat anti-biotin horseradish peroxidase (HRP) labelled antibody (dilution 1/1000) for 1 hour before thoroughly washing as previously described.
- 5 The membranes were then developed using Super Signal West Pico chemiluminescence substrate luminol and enhancer (Pierce) for 5 minutes prior to chemiluminescence detection reaction and exposure to X-ray film. The data are shown in Figure 8.
- Additionally, Western blots of all four constructs was performed using an anti-Penta Histidine monoclonal antibody according to the manufacturer's instructions for the
- 10 antibody (QIAGEN). The results are shown in Figure 9.
- **Refolding of the four Domain 4 fusion protein constructs**
- Refolding of the four constructs is necessary as they are purified under denaturing conditions. Refolding was performed by dialysis. The total protein concentration of the purified denatured protein was determined by BioRad protein assay and thereafter dialysed
- 15 against a 50 x volume of dialysis buffer containing 20 mM Tris-HCl, pH 7.4 at 4°C for 4 hours with constant rotation. Thereafter the buffer was changed and the fusion protein dialysed overnight at 4 °C. The protein concentration after refolding was measured by the BioRad protein assay according to manufacturer's instructions. Refolding was confirmed by Circular Dichroism (CD) analysis (data not shown).
- 20 All four Domain 4 variants were particularly hydrophobic and tended to fall out of solution during prolonged storage at 4°C. Out of all four constructs, constructs 1 and 2 were the least problematic, as they remained in solution even at high concentrations for few days. Construct 0 tended to precipitate very soon after refolding and concentration while construct 3 was not reliably expressed at all times.
- 25 The hydrophobic properties of the *E.coli*-produced Domain 4 variants may be due to lack of glycosylation. Peptide mapping experiments of sFLT-1 performed herein were consistent with one of the two potential glycosylation sites being glycosylated within Domain 4. Domain 4 contains two consensus N-glycosylation sites, 402NYT404 and 417NLT419; the peptide maps were consistent with 417NLT419 being consistently
- 30 glycosylated. Interestingly, this site lies within a particularly hydrophobic region of Domain 4.

Example 3: Analysis of FLT-1 Domain 4 molecules

Binding of Domain 4 on sFLT-1 is determined using Biomolecular Interaction Analysis (Biacore). (Fisher R.J. and Fivash, M. (1994) Curr. Opin. Biotech. 5, 389-395).

A monoclonal antibody (2B2) specific to Domains 1 to 3 of sFLT-1, but not Domain 4, 5 was immobilized on the sensor chip at a concentration of 30 μ g/ml in 10 mM NaAc, pH5. 50 μ g/ml of the four Domain 4 constructs in 20 mM Tris-HCl, pH7.4 were individually pre-incubated overnight at 4 °C with 50 nM sFLT-1 in HEPES buffer (0.01M Hepes, pH7.4, 10 0.15M NaCl, 3 mM EDTA, 0.005 % polysorbate 20 (v/v)). Thereafter the pre-incubated suspensions were passed across the immobilized monoclonal antibody and binding monitored by mass sensitive detection, measured in response units (RU). A control of 15 50nM sFLT-1 pre-incubated overnight in HEPES buffer containing no Domain 4 was also passed across the sensor chip and its binding affinity determined. A further control was also run whereby the four Domain 4 constructs, which have not been pre-incubated with sFLT-1, were passed over the immobilized monoclonal antibody. After each run the 15 sensor chip was regenerated in 10mM Glycine pH1.75 buffer ready for the next experiment.

Figures 10 and 11 illustrate the results obtained for the four Domain 4 constructs pre-incubated with sFLT-1 prior to passing over the immobilized monoclonal antibody. In conclusion, the BIACore data have shown that pre-incubation of sFLT-1 with either of 20 Domain 4 constructs 1, 2 or 3 result in inhibition of sFLT-1 binding to the sensor chip (Table 1). In contrast, pre-incubation with construct 0 showed very poor inhibition (Table 1). These results clearly indicate that constructs 1, 2 and 3 interact with sFLT-1, preventing 25 its interaction with the monoclonal antibody immobilized on the sensor chip. This interaction is a specific domain 4-sFLT-1 interaction and is not due to direct binding competition of the Domain 4 construct to the anti-body immobilised on the chip. The data in Figure 11 have confirmed that none of the constructs bind directly onto the chip.

Alternatively, the FLT-1 molecule may be immobilised on the sensor chip and the binding affinity determined by measuring the response at the course of time at increasing concentrations of the Domain 4 protein in the presence and absence of VEGF.

30 The dimeric/monomeric state of Domain 4 as well as its interactions with sFLT-1 in solution may also be determined by gel filtration where molecules are fractionated by size.

High resolution gel filtration is used for separating the monomers from the dimers and heterodimers. Suitable columns are Sephadryl S-100 HR and S-200 HR (Pharmacia Biotech).

Direct binding of Domain 4 on the full-length receptor on the cell surface may be
5 determined by receptor-binding experiments, where radioactively or fluorescent-labelled
Domain 4 binds on HUVEC cells grown to confluence in 24-well tissue culture plates
under the appropriate culture conditions. For competition binding studies, labelled Domain
4 may be mixed with various concentrations of unlabelled sFLT-1. After a few hours'
incubation on ice, cells are washed 4 times with medium. Bound labelled Domain 4 is
10 removed from cells by lysis with 0.1% SDS, and counts are measured in a γ counter.

The HUVEC proliferation assay (Clauss *et al.*, 1996, J. Biol. Chem. 271 (3): 17629-17634)
may be also used to determine the inhibitory effect of the Domain 4 protein molecules on
receptor dimerisation and subsequent cell proliferation. HUVECs are seeded in growth
medium (EBM containing 2%FBS and GA-1000) at 5000 cells/well in 96-well cluster
15 plates. The plates are left overnight for cell attachment/stabilisation and the cells are then
treated with VEGF in the presence and absence of Domain 4 molecules at variable
concentrations. Cell proliferation is measured 48hrs after incubation by the Br-dU ELISA
(Boehringer Mannheim) (Porstmann *et al.*, (1985) J. Immunol. Methods 82: 169-179).

Example 4: Preparation of a monoclonal antibody against Domain 4

20 Alternatively, an antibody, preferably a monoclonal antibody, recognising Domain 4 may
be used to restrict dimerisation of the receptor. One antibody recognising Domain 4 has
been produced herein and the method for its production is described below.

Three hosts were immunised with sFLT-1 produced in CHO cells in Freund's complete
adjuvant, intra-muscular, followed by 3 immunisations at 2 week intervals in Freund's
25 incomplete adjuvant subcutaneously, followed by a test bleed 1 week later each
immunisation. A final intra-peritoneal immunisation was given 2 weeks later before
spleen collection. 1 of the 3 hosts was chosen for fusion and 25 hybridomas were initially
selected. 6 hybrids were selected for cloning and 3 clones from each hybridoma were
transferred into larger plates and yielded sufficient cells for frozen storage.

30 The initial bleeds were screened on a 96-well plate being coated overnight with sFLT-1,
2 μ g/ml in PBS, 100 μ l/well at 4°C. The plate was blocked with 1% BSA in PBS pH 7.4

(200µl/well, 60min, 37°C). Primary incubation was performed with 50µl anti-serum diluted in assay buffer plus 50µl competing antigen (MP9814 at 5µg/ml in assay buffer) for 1hr at 37°C. Goat anti-mouse-IgG-alkaline phosphatase labelled (1:3000 dilution) was used for detection of the primary antibody bound onto the plate. The amount of labelled antibody bound was determined by an alkaline phosphatase assay, using 0.5mg/ml pNPP substrate in 9.7% diethanolamine, pH 9.6.

Hybridoma screening was performed as described for the screening of the test bleeds with the exception that undiluted hybridoma supernatant was used during the primary incubation in place of serum. Five hybridomas were positive for binding to sFLT-1, 5G10, 6C5, 15F8, 1H11 and 7C10 and they were screened against a number of sFLT-1 variants for binding on dot and slot blots (see Figure 12).

Dot Blot Results

Hybridoma	5G10	6C5	15F8	1H11	7C10	
Protein	conc. / neat					
sFLT-1 Dom 1-5 crude	40 µg/ml	✓	✓	low	✓	✓
sFLT-1 Dom 1-5 purified	40 µg/ml	✓	✓	low	✓	✓
sFLT-1 Dom 1-3 crude	40 µg/ml (?)	low	✗	✗	✗	low
sFLT-1 Dom1-3 partially purified	40 µg/ml (?)	low	✗	✗	✗	?
sFLT-1 Dom 1-4/5 crude	neat	✓	✓	v.v. low	✓	✓
sFLT-1 Dom 2-3	neat	✓	✗	✗	✗	v. low
sFLT-1 Dom 2 fusion	neat	✓	✗	✗	✗	✓
sFLT-1 Dom. 4 (construct 0)	neat	✗	✗	✗	✓	✗
Blank media Excel 302	neat	✗	✗	✗	✗	✗

Blank media GMEM (1% serum)	neat	x	x	x	x	x
Wild type CHO K1 media	neat	x	x	x	x	x
Mock transfected media	neat	x	x	x	x	x

All samples were loaded at volumes of 1 μ l, 5 μ l, 12.5 μ l and 25 μ l at the above concentrations, onto a nitrocellulose membrane. The membranes were blocked in a 5% skimmed milk powder solution overnight at 4°C. Primary hybridomas were prepared in
5 5% skimmed milk powder incubation buffer and secondary antibody was added at an 1:1000 dilution in 5% skimmed milk powder incubation buffer.

Hybridoma 1H11 is positive for Domain 4 (Construct 0). This hybridoma as well as hybridomas 6C5, 18G12 and 15F8 were tested for its affinity to the variants of Domain 4 by slot blots. All Domain 4 constructs tested were positive for hybridoma 1H11 while
10 hybridomas 6C5, 18G12 and 15F8 did not cross-react with any of the Domain 4 constructs and the results are shown in Figure 12.

Table 1: Preferred constructs derived from the FLT-1, FLK, KDR and FLT-4 constructs

<u>FLT-1</u>	<u>FLK</u>	<u>KDR</u>	<u>FLT-4</u>
316 to 406	314 to 404	312 to 402	315 to 403
316 to 407	314 to 405	312 to 403	315 to 404
316 to 408	314 to 406	312 to 404	315 to 405
316 to 409	314 to 407	312 to 405	315 to 406
316 to 410	314 to 408	312 to 406	315 to 407
316 to 411	314 to 409	312 to 407	315 to 408
316 to 412	314 to 410	312 to 408	315 to 409
316 to 413	314 to 411	312 to 409	315 to 410
316 to 414	314 to 412	312 to 410	315 to 411
316 to 415	314 to 413	312 to 411	315 to 412
316 to 416	314 to 414	312 to 412	315 to 413
316 to 417	314 to 415	312 to 413	315 to 414
316 to 418	314 to 416	312 to 414	315 to 415
316 to 419	314 to 417	312 to 415	315 to 416
316 to 420	314 to 418	312 to 416	315 to 417
316 to 421	314 to 419	312 to 417	315 to 418
316 to 422	314 to 420	312 to 418	315 to 419
316 to 423	314 to 421	312 to 419	315 to 420
316 to 424	314 to 422	312 to 420	315 to 421
316 to 425	314 to 423	312 to 421	315 to 422
316 to 426	314 to 424	312 to 422	315 to 423
316 to 427	314 to 425	312 to 423	315 to 424
316 to 428	314 to 426	312 to 424	315 to 425
316 to 429	314 to 427	312 to 425	315 to 426
316 to 430	314 to 428	312 to 426	315 to 427
316 to 431	314 to 429	312 to 427	315 to 428
316 to 432	314 to 430	312 to 428	315 to 429
316 to 433	314 to 431	312 to 429	315 to 430
316 to 434	314 to 432	312 to 430	315 to 431
316 to 435	314 to 433	312 to 431	315 to 432

316 to 436	314 to 434	312 to 432	315 to 433
316 to 437	314 to 435	312 to 433	315 to 434
316 to 438	314 to 436	312 to 434	315 to 435
316 to 439	314 to 437	312 to 435	315 to 436
316 to 440	314 to 438	312 to 436	315 to 437
316 to 441	314 to 439	312 to 437	316 to 403
316 to 442	314 to 440	312 to 438	316 to 404
316 to 443	315 to 404	313 to 402	316 to 405
316 to 444	315 to 405	313 to 403	316 to 406
316 to 445	315 to 406	313 to 404	316 to 407
316 to 446	315 to 407	313 to 405	316 to 408
316 to 447	315 to 408	313 to 406	316 to 409
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339 to 447	341 to 412	339 to 410	343 to 430
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340 to 407	341 to 414	339 to 412	343 to 432
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Table 2: Percentage inhibition of sFLT-1 binding to a monoclonal antibody (2B2) after pre-incubation with 50 µg/ml Domain 4 constructs 0, 1, and 2 and 25 µg/ml Domain 4 construct 3 as determined by BIACore analysis.

Sample	% inhibition of sFLT-1
Domain 4 construct 0	5.85 %
Domain 4 construct 1	64.66 %
Domain 4 construct 2	73.35 %
Domain 4 construct 3	44.87 %

CLAIMS

1. A protein consisting of the amino acid sequence of the fourth Ig-like domain of a VEGF receptor, or a variant of said protein that retains the ability to bind to a VEGF receptor.
- 5 2. A protein according to claim 1, wherein said VEGF receptor belongs to either the FLT receptor family or the KDR/FLK receptor family.
3. A protein according to claim 2, wherein said VEGF receptor is the FLT-1 receptor, the KDR/FLK receptor or the FLT-4 receptor.
4. A protein according to any one of the preceding claims, wherein said VEGF receptor is 10 a human VEGF receptor.
5. A protein according to any one of the preceding claims, wherein said protein binds to the fourth Ig-like domain of a VEGF receptor
6. A protein according to any one of the preceding claims that binds to the fourth Ig-like domain of a VEGF receptor with a dissociation constant of $2\mu M$ or less, preferably, 15 $0.2\mu M$ or less, more preferably $2nM$ or less, even more preferably, $20pm$ or less.
7. A protein according to any one of the preceding claims, wherein said fourth Ig-like domain comprises at least residues 344-406 of the full length FLT-1 sequence given in Figure 1, but no more than residues 316-447 of this sequence.
8. A protein according to claim 7 which consists of residues 338-440, 330-429, 330-440 20 or 338-429 of the full length FLT-1 sequence or a variant of this sequence containing one or more amino acid substitutions that do not decrease the binding affinity of the protein for the fourth Ig-like domain of a VEGF receptor.
9. A protein according to any one of claims 1-6, wherein said fourth Ig-like domain comprises at least residues 342-404 of the full length FLK sequence given in Figure 2, 25 but no more than residues 314-440 of this sequence.
10. A protein according to claim 9 which consists of residues 335-435, 328-424, 328-435 or 335-424 of the full length FLK sequence or a variant of this sequence containing one or more amino acid substitutions that do not decrease the binding affinity of the protein for the fourth Ig-like domain of a VEGF receptor.

11. A protein according to any one of claims 1-6, wherein said fourth Ig-like domain comprises at least residues 340 to 402 of the full length KDR sequence given in Figure 3, but no more than residues 312-438 of this sequence.
12. A protein according to claim 11 which consists of residues 333-433, 326-422, 326-433 or 333-422 of the full length KDR sequence or a variant of this sequence containing one or more amino acid substitutions that do not decrease the binding affinity of the protein for the fourth Ig-like domain of a VEGF receptor.
5
13. A protein according to any one of claims 1-6, wherein said fourth Ig-like domain comprises residues 343-403 of the full length FLT-4 sequence given in Figure 4, but no more than residues 315 to 437 of this sequence.
10
14. A protein according to claim 13 which consists of residues 339-437, 329-423, 329-437 or 339-423 of the full length FLT-4 sequence or a variant of this sequence containing one or more amino acid substitutions that do not decrease the binding affinity of the protein for the fourth Ig-like domain of a VEGF receptor.
15. A protein according to any one of the preceding claims that has been genetically or chemically fused to one or more peptides or polypeptides.
15
16. A protein according to claim 15, which comprises repeated amino acid sequences of the fourth Ig-like domain of a VEGF receptor.
17. A protein according to claim 15 or claim 16, fused to a label.
20. A protein according to any one of the preceding claims, for use as a pharmaceutical.
18
19. A functional equivalent of a protein according to any one of claims 1-17 that binds to the fourth Ig-like domain of a VEGF receptor.
20. A functional equivalent according to claim 19, which is an antiidiotypic antibody, a peptide, an oligopeptide, a peptidomimetic compound or a drug molecule, such as a small natural or synthetic organic molecule.
25
21. Use of a protein according to any one of claims 1-17 or a functional equivalent according to claim 19 in the manufacture of a medicament for the treatment of a disorder whose pathology is dependent upon a VEGF family-mediated pathway.
22. A nucleic acid encoding a protein according to any one of claims 1 to 17.

23. A vector comprising a nucleic acid according to claim 22.
24. A host cell comprising a vector according to claim 23.
25. A pharmaceutical composition comprising a protein according to any one of claims 1-17 or a functional equivalent according to claim 19 or 20, in association with a suitable pharmaceutical excipient.
5
26. A method of treating a patient suffering from a disorder whose pathology is dependent upon a VEGF-mediated pathway comprising administering to a patient a therapeutically-effective amount of a protein according to any one of claims 1-17, a functional equivalent according to claim 19 or 20, a nucleic acid according to claim 22, or a pharmaceutical composition according to claim 25.
10
27. A method according to claim 26, wherein said disorder is an inflammation, psoriasis, rheumatoid arthritis, hemangiomas, leiomyomas, diabetic retinopathy, angiofibromas, endometriosis, macular degeneration, retinal neovascularisation or cancer.
28. A transgenic animal that has been transformed by a nucleic acid molecule according to
15 claim 22 or a vector according to claim 23.
29. A method for inhibiting the dimerisation of a VEGF receptor, comprising bringing the receptor into contact with a protein, or functional equivalent according to any one of claims 1-20.

1/13

FIG. 1

FLT-1

1	MVSYWDTGVLLCALLSCLLTGSSSGSKLKDPESLKGQTQHIMQAGQTLH	50
51	LQCRGEAAHKWSLPEMVSKESELTSITKSACGRNGKQFCSTLNTAQAN	100
101	HTGFYSCKYLAVPTSKKETESAIIYIFISDTGRPFVEMYSEIPEIIHMTE	150
151	GRELVIPCRVTSPNITVTLKKFPLDTLIPDGKRIIWDSRKGFIISNATYK	200
201	EIGLLTCEATVNGHLYKTNYLTHRQNTIIDVQISTPRPVKLLRGHTLVL	250
251	NCTATTPLNTRVQMTWSYPDEKNKRASVRRRIDQSNSHANIFYSVLTIDK	300
301	MQNKDKGLYTCRVRSGPSFKSVNTSVHIYDKAFITVKHRKQQVLETVAGK	350
351	RSYRLSMKVKAFPSPEVVWLKDGLPATEKSARYLTRGLYSLIIKDVTEEDA	400
401	GNYTILLSIKQSNVFKNLTATLIVNVKPQIYEKAVSSFPDPALYPLGSRQ	450
451	ILTCTAYGIPQPTIKWFWHPCNHNHSEARCDFCSNNEESFILDADSNMGN	500
501	RIESITQRMAIEGKNKMASTLVVADSRIISGIYICIASNKVGTVGRNISF	550
551	YITDVPNGFHVNLEKMPTEGEDLKLSCTVNKFLYRDVTWILLRTVNNRTM	600
601	HYSISKQKMAITKEHSITLNLTIMNVSLQDSGYACRARNVYTGEEILQK	650
651	KKEITIRDQEAPYLLRNLSDHTVAISSSTLDCHANGVPEPQITWFKNHH	700
701	KIQQEPMIILGPGSSTLFIERVTEEDEGVYHCKATNQKGSVESSAYLTVQ	750
751	GTSDKSNLIELITLTCTCVAATLFWLLTLLIRKMKRSSSEIKTDYLSIIM	800
801	DPDEVPLDEQCERLPYDASKWEFARERLKLGKSLGRGAFGKVVQASAFCGI	850
851	KKSPTCRTAVKMLKEGATASEYKALMTELKILTHIGHHLNVNLLGACT	900
901	KQGGPLMVIVEYCKYGNLSNYLKSKRDLFFLNKDAALHMEPKKEKMEPGL	950
951	EQGKKPRLDSVTSESFASSGFQEDKSLSDVEEEEDSDGFYKEPITMEDL	1000
1001	ISYSFQVARGMEFLSSRKCIHRDLAARNILLSENNVVKICDFGLARDIYK	1050
1051	NPDYVRKGDRPLKWMAPESIFDKIYSTKSDVWSYGVLLWEIFSLGGSP	1100
1101	YPGVQMDEDPCSRLREGMRMRAPEYSTPEIYQIMLDCWHRDPKERPRFAE	1150
1151	LVEKLGDLLQANVQQDGKDYIPINAILTGNSGFTYSTPAFSEDFFKESIS	1200
1201	APKFNSGSSDDVRYVNAFKFMSLERIKTFEELPNATSMFDDYQGDSSTL	1250
1251	LASPMLKRFTWTDSPKASLKIDLRTSKSKESGLSDVSRPSFCHSSCGH	1300
1301	VSEGKRRFTYDHAELEKIACCSPPPDYNSSVVLYSTPPI	1339

2/13

FIG. 2

FLK

1	MESKALLAVALWFCVETRAASVGLTGFHPPKLSTQKDILTLANTTLQ	50
51	ITCRGQRDLDWLWPNAQRDSEERVLTVECGGGDSIFCKTLTIPRVVGNDT	100
101	GAYKCSYRDVDIASTVYVYVRDYRSPFIASVSDQHGIVYITENKNKTVVI	150
151	PCRGSIISNLNVSLCARYPEKRFVPDGKRISWDSEIGFTLPSYMISYAGMV	200
201	FCEAKINDETYQSIMYIVVVVGYRIYDVILSPPHEIELSAGEKLVLNCTA	250
251	RTELNVGLDFTHSPPSKSHKKIVNRDVKPFPGTVAKMFLSTLTIESVT	300
301	KSDQGEYTCVASSGRMIKRNRFTFVRVHTKPFIAFGSGMKSLSVEATVGSQV	350
351	RIPVKYLSYPAPDIKWYRNGRPIESNYTMIVGDELTIMEVTERDAGNYTV	400
401	ILTNPISMEKQSHMVSLLVNVPPQIGEKALISPMDSYQYGTMQTLTCTVY	450
451	ANPPLHHIQWYWQLEEACSYRPQQTSPYACKEWRHVEDFQGGNKIEVTKN	500
501	QYALIEGKNKTVSTLVIQAANVSALYKCEAINKAGRGERVISFHVIRGPE	550
551	ITVQPAAPTEQESVSLCTADRNTFENLTWYKLGSQATSVHMGESLTPV	600
601	CKNLDALWKLNGTMFSNSTNDILIVAFQNDSLQDQGDYVCSAQDKTKKR	650
651	HCLVKQLIILERMAPMITGNLENQTTIGETIEVTCPASGNPTPHITWFK	700
701	DNETLVEDSGIVLRDGNRNLTIIRRVRKEDGGLYTCQACNVLGCARAETLF	750
751	IIEGAQEKTNLEVIILVGTAVIAMFFWLLLIVLRTVKRANEGETKTGYL	800
801	SIVMDPDELPLDERCERLPYDASKWEFPRDRKLKGPLGRGAFGQVIEAD	850
851	AFGIDKTATCKTAVKMLKEGATHSEHRALMSELKILIHIGHHLNVVNLL	900
901	GACTKPGGPLMVIVEFKFGNLSTYLRGKRNEFVPYKSKGARFRQGKDYV	950
951	GELSVDLKRR LDSITSSQSSASSGFVEEKSLSDVEEEEASEELYKDFLTL	1000
1001	EHLICYSFQVAKGMELASRKCIHRDLAARNILLSEKNVKICDFGLARD	1050
1051	IYKDPDYVRKG达尔PKWMAPETIFDRVYTIQSDVWSFGVLLWEIFSL	1100
1101	GASPYPGVKIDEFCRRLKEGTRMRAPDYTTPEMYQTMLDCWHEDPNQR	1150
1151	PSFSELVEH LGNLLQANAQQDGKDYIVLPMS SETLSMEEDGLSLPTSPV	1200
1201	SCMEEEVCDPKFHYDNTAGISHYLQNSKRKSRPVSVKTFEDI PLEEPE	1250
1251	VKVIPDDSQTDGMVLASEELKTLEDRNKLSPSF GGMMPSKSRESVASE	1300
1301	GSNQTSGYQSGYHSDDTTVYSSDEAGLLK MVDAAVHADSGTTLQLTS	1350
1351	CLNGSGPVPAPPPTPGNHERGAA 1373	

3/13

FIG. 3

KDR

1	MESKVLLAVALWLCVETRAASVGLPSVSLDLPLRSIQKDILTIKANTTLQ	50
51	ITCRGQRDLDWLWPNNQSGSEQRVEVTECSDGLFCCKTLTIPKIVGNDTGA	100
101	YKCFYRETDLASVIYVVQDYRSPFIASVSDQHGVVYITENKNKTVVIPC	150
151	LGSISNLNVSLCARYPEKRFVPDGNRISWDSKKGFTIPSYMISYAGMVFC	200
201	EAKINDESYQSIMYIVVVVGYRIYDVVLSPSHGIELSVGEKLVLNCTART	250
251	ELNVGIDFNWEYPSSKHQHKKLVNRLKTQSGSEMKKFLSTLTIDGVTRS	300
301	DQGLYTCAASSGLMTKNSTFVRVHEKPFVAFGSGMESLVEATVGERVRI	350
351	PAKYLGYPPPEIKWYKNGIPLESNHTIKAGHVLTIMEVSERDTGNYTVIL	400
401	TNPISKEKQSHVVSLVVYVPPQIGEKSЛИSPDSYQYGTQTLCVYAI	450
451	PPPPIIHWYWQLEEECANEPSQAVSVTNPYPCEEWRSEDFQGGNKIEVN	500
501	KNQFALIEGKNKTVSTLVIQAANVSALYKCEAVNKVGRGERVISFHVTRG	550
551	PEITLQPDMQPTEQESVSLWCTADRSTFENLTWYKLGPKPLPIHVGELPT	600
601	PVCKNLDTLWKLNAATMFSNSTNDILIMELKNASLQDQGDYVCLAQDRKTK	650
651	KRHCVVRQLTVLERVAPTTGNLENQTTSIGESIEVSCTASGNPPPQIMW	700
701	FKDNETLVEDSGIVLKDGNRNLTIRRVRKEDEGLYTCQACSVLGCAKVEA	750
751	FFIIEGAQEKTNLEIIIILVGTAVIAMFFWLLVIIIRTVKRANGGELKTG	800
801	YLSIVMDPDELPLDEHCERLPYDASKWEFPRDRLKLGPKLGRGAFGQVIE	850
851	ADAFGIDKTATCRTAVKMLKEGATHSEHRALMSELKILIHIGHHHLNVVN	900
901	LLGACTKPGGPLMVIVEFCKFGNLSTYLRSKNEFVPYKTKGARFRQGKD	950
951	YVGAI PVDLKRRLLDSITSSQSSASSGFVEEKSLSDVEEEEAPEDLYKDFL	1000
1001	TLEHLICYSFQVAKGMELASRKCIHRDLAARNILLSEKNVKICDFGLA	1050
1051	RDIYKDPDYVRKGDLPLKWMAPETIFDRVYTIQSDVWSFGVLLWEIFS	1100
1101	LGASPYPGVKIDEFCRRLKEGTRMRAPDYTTPEMYQTMLDCWHGEPSQR	1150
1151	PTFSELVEHGNLLQANAQQDGKDYIVLPISETLSMEEDSGLSLPTSPVS	1200
1201	CMEEEVCDPKFHYDNTAGISQYLQNSKRKSRPVSVKTFEDIPLLEEPEVK	1250
1251	VIPDDNQTDGMVLASEELKTLEDRTKLSPSFGGMVPSKSRESVASEGSN	1300
1301	QTSGYQSGYHSDDTDTTVYSSEEELLKIEIGVQTGSTAQILQPDSGTT	1350
1351	LSSPPV 1356	

4/13

FIG. 4

FLT4

1	MQRGAALCLRLWLCLGLLDGLVSDYSMTPPTLNITEESHVIDTGDLSIS	50
51	CRGQHPLEWAWPGAQEAPATGDKDSEDTGVVRDCEGTDARPYCKVLLHE	100
101	VHANDTGSYVCYYKYIKARIEGTTAASSYVFVRDFEQPFINKPDPLLVR	150
151	KDAMWVPCLVSIPGLNVTLRSQSSVLWPDGQEVVWDDRRGMLVSTPLLHD	200
201	ALYLQCETTWGDQDFLSNPFLVHITGNELYDIQLLPRKSLELLVGEKLV	250
251	NCTVWAEFNSGVTFDWDYPGKQAERGKWPERRSQQTTELSSILTIHNV	300
301	SQHDLGSYVCKANNGIQRFRESTEVIVHENPFISVEWLKGPILEATAGDE	350
351	LVKLPVKLAAYPPPEFQWYKDGKALSGRHSPhALVLKEVTEASTGTYTLA	400
401	LWNSAAGLRRNISLELVNVPPQIHEKEASSPSIYSRHSRQALTCTAYGV	450
451	PLPLSIQWHWRPWTPCKMFAQRSLRRQQQDLMPQCRDWRAVTTQDAVNP	500
501	IESLDTWTEFVEGKNKTVSKLVIQNANVSAMYKCVVSNKVGQDERLIYFY	550
551	VTTIPDGFTIESKPSEELLEGQPVLLSCQADSYKYEHLRWYRLNLSTLHD	600
601	AHGNPLLLDCNVHLFATPLAASLEEAVPGARHATLSLSIPRVAPEHEGH	650
651	YVCEVQDRRSHDKHCHKKYLSVQALEAPRLTQNLTDLLVNVSDSLEMQCL	700
701	VAGAHAPSIVWYKDERLLEEKSGVDLADSNQKLSIQRVREEDAGPYLCV	750
751	CRPKGCVNSSASAVEGSEDKGSMEIVILVGTGVIAVFFWVLLLLIFCNM	800
801	RRPAHADIKTGYLSIIMDPGEVPLEEQCEYLSYDASQWEFPRERLHLGRV	850
851	LGYGAFGVVEASAFGIHKGSSCDTVAVKMLKEGATASEQRALMSELKIL	900
901	IHIGNHNVNVNLLGACTKPQGPLMVIVEFCKYGNLSNFLRAKRDAFSPCA	950
951	EKSPEQRGRFRAMVELARLDRRRGSSDRVLFARFSKTEGGARRASPDQE	1000
1001	AEDLWLSPLTMEDLVCYSFQVARGMEFLASRKCIHRDLAARNILLSESDV	1050
1051	VKICDFGLARDIYKDPDYVRKGSARLPLKWMAPESIFDKVYTTQSDVWSF	1100
1101	GVLLWEIFSLGASPYPGVQINEEFCQRVRDGTRMRAPELATPAIRHIMLN	1150
1151	CWSGDPKARPAPSDLVEILGDLLQGRGLQEEEVCMAPRSSQSSEEGSFS	1200
1201	QVSTMALHIAQADAEDSPPSLQRHSLAARYYNWVSFPGCLARGAETRGSS	1250
1251	RMKTFEFPMTPTTYKGSDVNQTDGMVLASEEFQIESRHRQESGFR	1298

5/13

FIG. 5

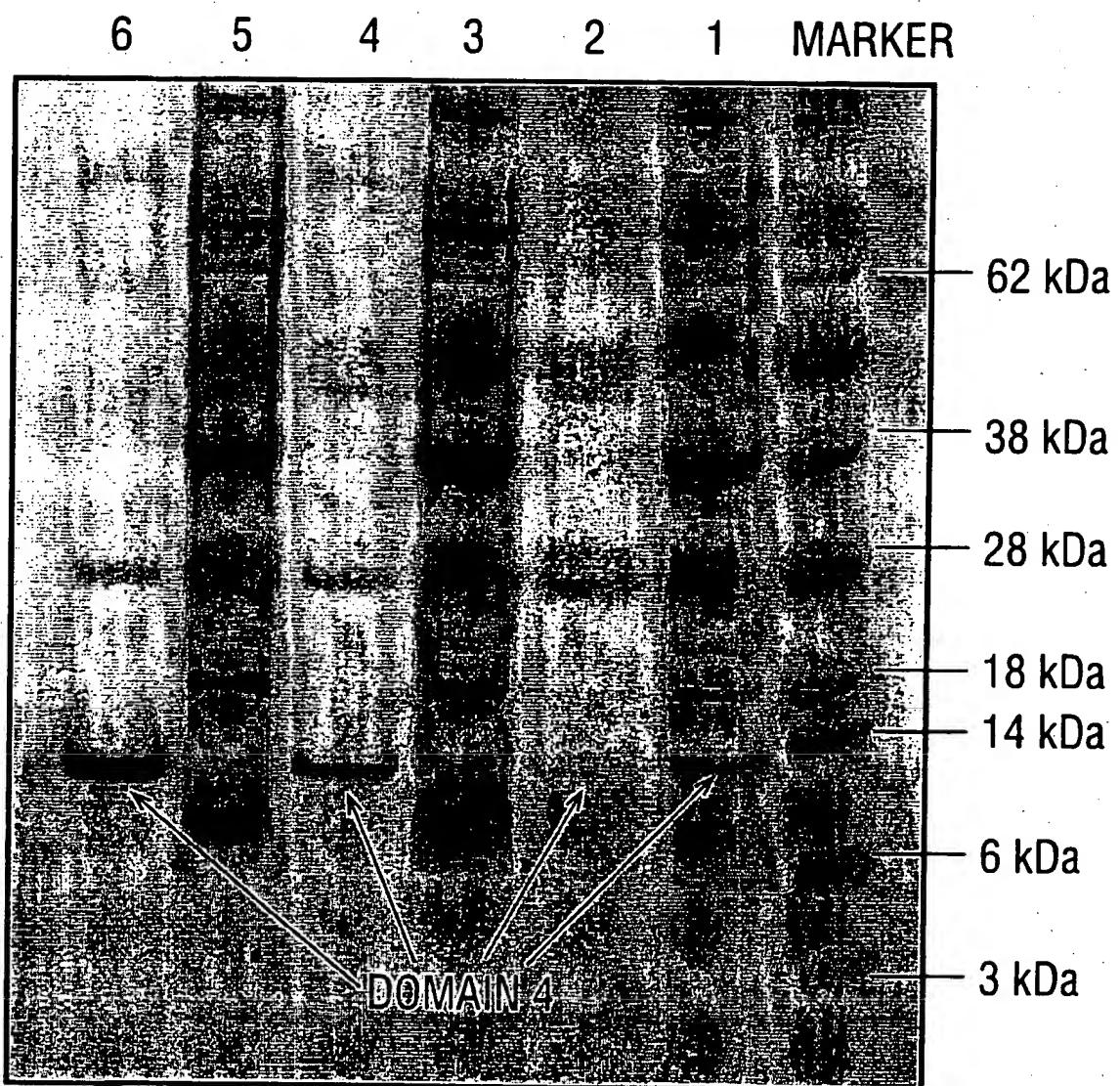
FLT	316	GPSFKSVNTSVHIY	330DKAFITVKHRKQOQVLE	-TVAGKRSYRLLSMKVKAFPSPEVVWLIKDGGLPATEKSARYLTR	
KDR	312	GLMTKKNSTFVRVH	326EKPFVAFGSGMESLVEATV	-GER-VRIPAKYLGYPPEIKWYKNGIP-LESN-HTIKA	
FLK	314	GRMIKRNRRTFVRVH	328TKPFIAGSGMKSJLVEATV	-GSQ-VRIPVKYLSYPAPDIKWIYRNNGRP-IESNYTMI-V	
FLT4	315	GIQRFRESTEVIH	329ENPFISVEWLKGPILEATA	-GDELVKLPVKLAAYPPPEFQWYKDG-----KALSGRHS	
FLT		GYSLIKIDVTEEDAGNYTILL	--SI---KQSNVFKNLTATLIVNKQIYEKAVSSFPD	440 PALYPLG447	
KDR		GHVLTIMEVSERDTGNYTVLITNPISKEKQSHV	-----SLVVYVPPQIGEKSLLISPVD	433 SYQY--G438	
FLK		GDELTIMEVTERDAGNYTVLITNPISMEKQSHMV	-----SLVNVPPQIGEKALLSPMD	435 SYQY--G440	
FLT4		PHALVLUKEVTEASTGTYTTLALWNSAAGLR	RNISLELVNNVPPQIHEKEASSPS-	433 IYSR---437	

Underlined:

Construct 0

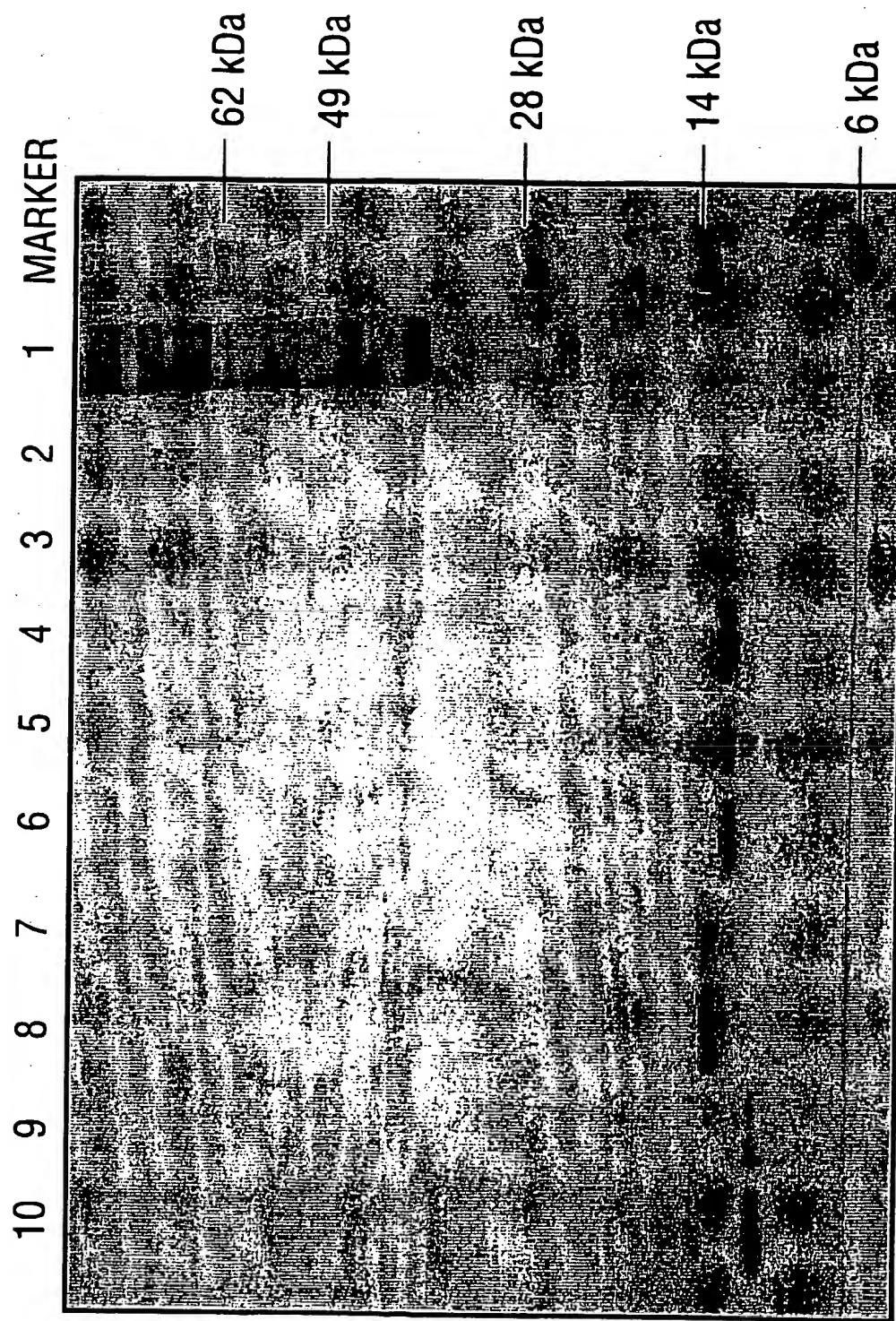
6/13

FIG. 6



7/13

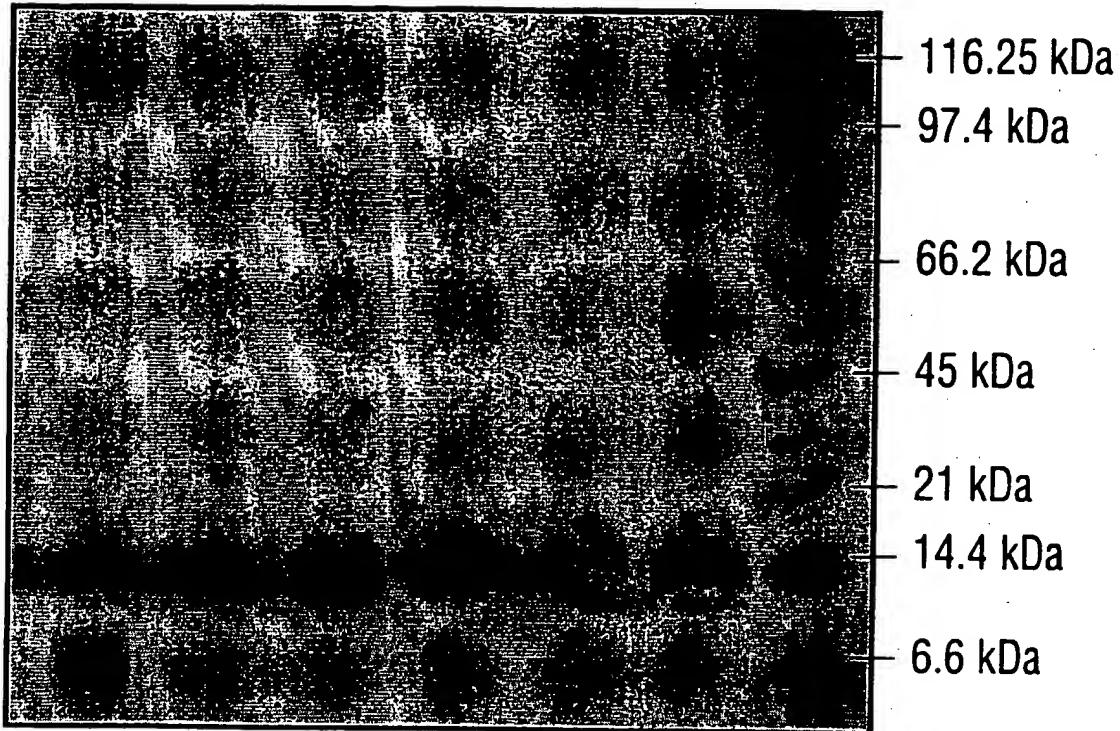
FIG. 7



8/13

FIG. 8

1 2 3 4 5 6 7 8 MARKER



MARKER 1 2 3 4

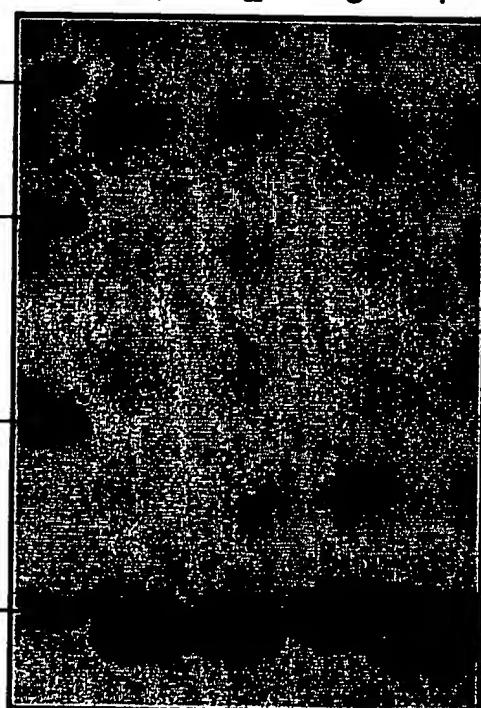
75 kDa

50 kDa

30 kDa

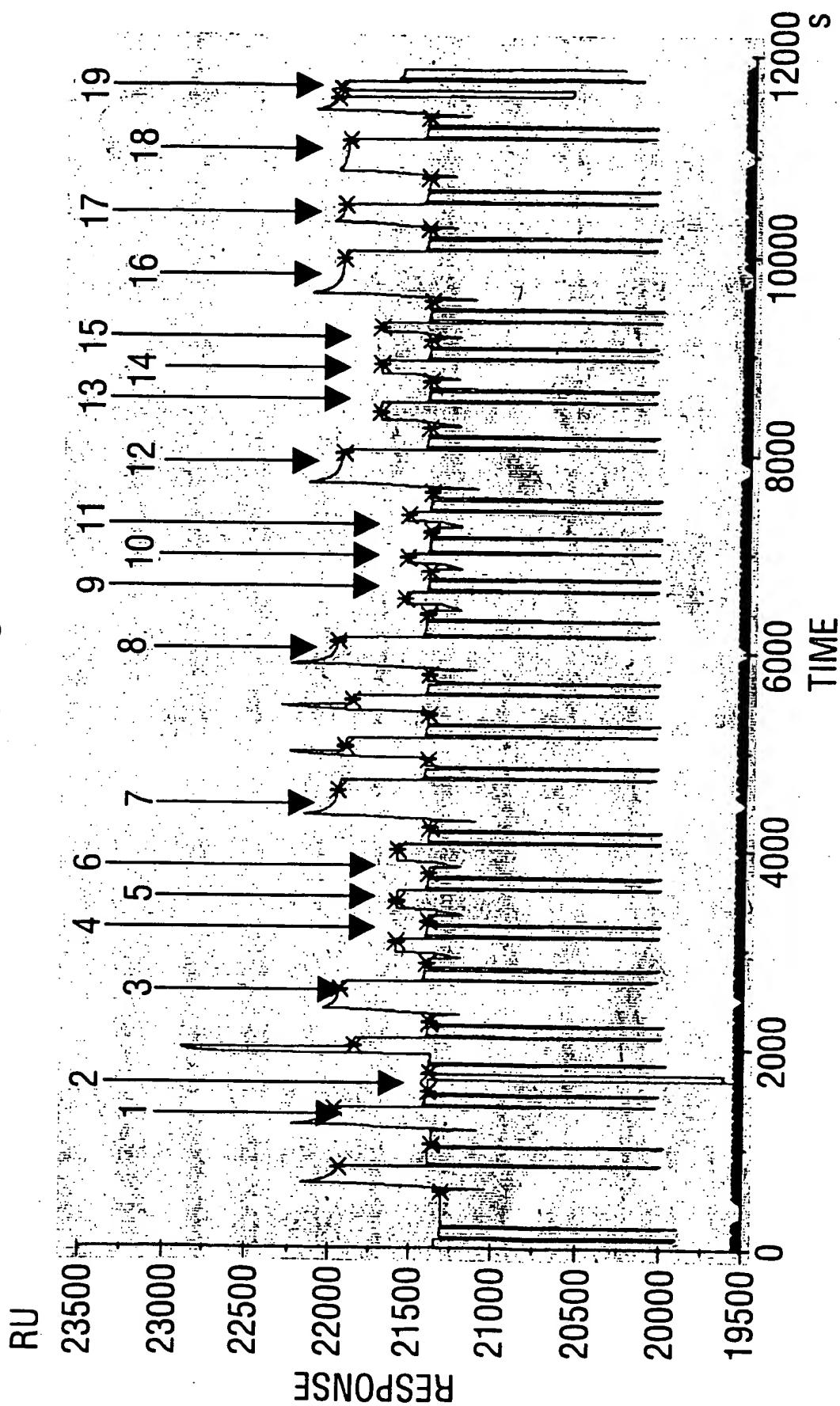
15 kDa

FIG. 9



9/13

FIG. 10



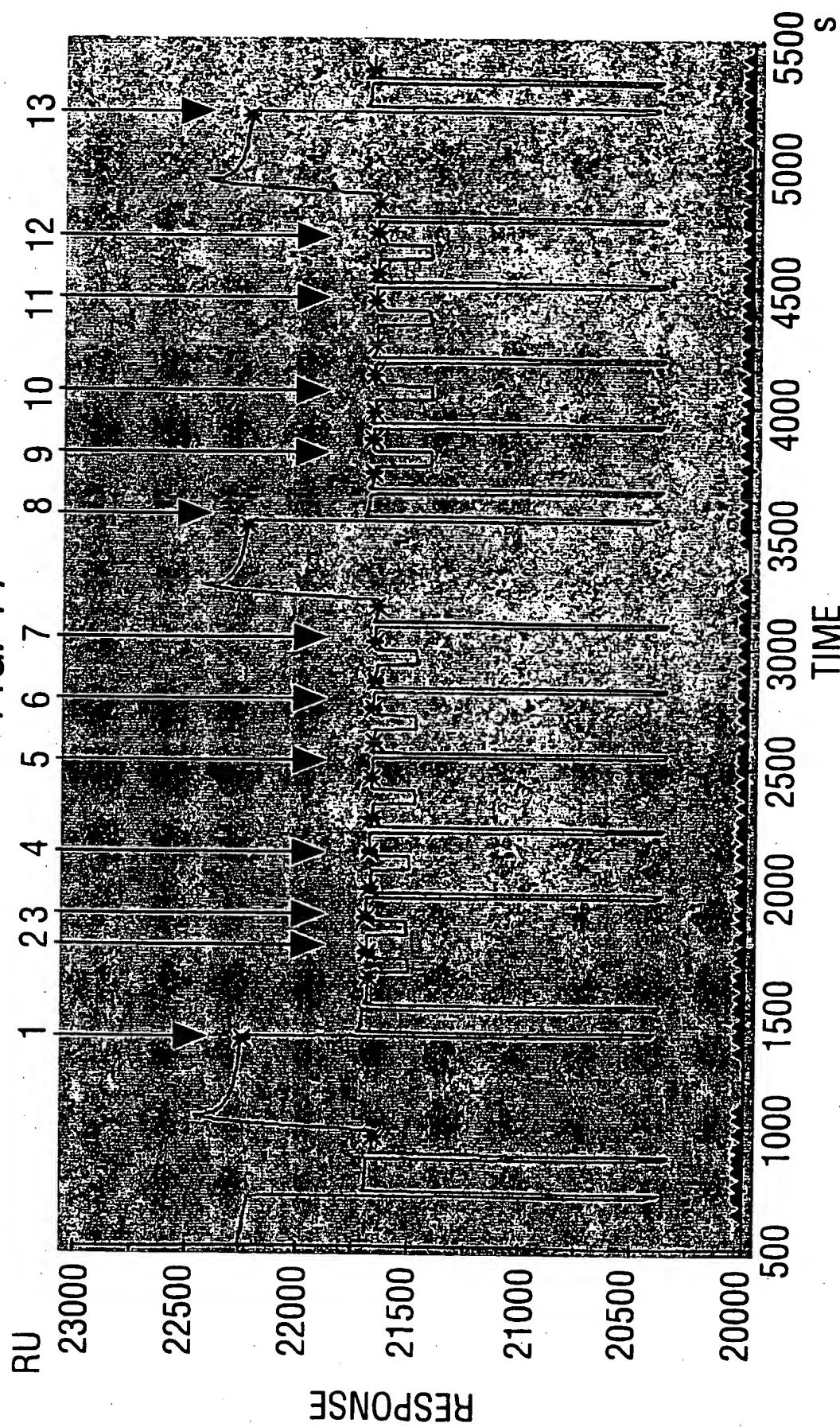
10/13

FIG. 10(*contd.*)

Number from Figure 5	50 nM sFLT	20 mM Tris- HCl, pH7.4	50 nM sFLT + 50 µg/ml D4 (0)	50 nM sFLT + 50 µg/ml D4 (1)	50 nM sFLT + 50 µg/ml D4 (2)	50 nM sFLT + 25 µg/ml D4 (3)
1	540					
2		- 10.9				
3			553.8			
4				194.3		
5					195.6	
6						193.2
7		566.8				
8		561.5				
9					137.2	
10						138.9
11						138.1
12		545.3				
13						
14						306
15						300.5
16		535.2				303.2
17						
18					514.3	
19					489.8	
						555

11/13

FIG. 11



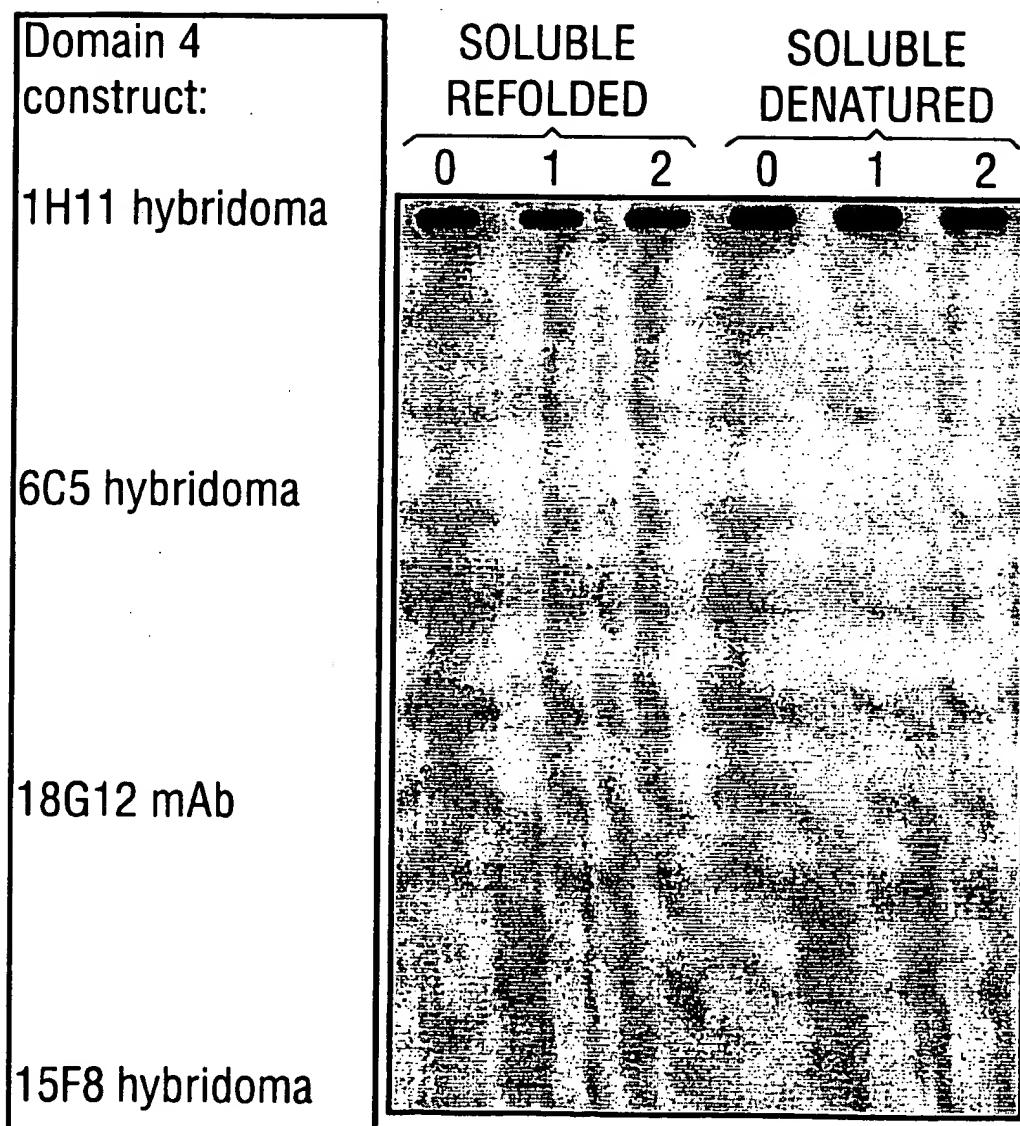
12/13

FIG. 11 (contd.)

Number from Figure 1	50 nM sFLT	50 µg/ml D4 (0)	50 µg/ml D4 (1)	50 µg/ml D4 (2)	25 µg/ml D4 (3)
1	584.8				
2		4.7			
3		13.1			
4		0.7			
5			3.1		
6				5	
7				5.9	
8	578.1				
9					3.3
10					1.8
11					2.1
12					-1
13	569.3				

13/13

FIG. 12



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(Continued on next page)

(54) Title: PROTEIN COMPRISING IG-LIKE DOMAIN 4 OF A VEGF RECEPTOR MEDIATES RECEPTOR DIMERIZATION,
BUT NOT ACTIVATION

PLK
1 MESKALLAVALWPCVETRAASVGLTGDFLHPPKLSTQKDILTLANTTLQ 50
51 ITCRGQRDLWLPNAQRDSEERVLVTECGGGDSIFCKTLTIPRVRVGNDT 100
101 GAYKCSYRDVDIASTVVYVVRDYRSPIASVSDQHGIVVYITENKNKTVV 150
151 PCRGSIISNLNVSLCARYPEKRFVPDGNRISWDSEIGFTLPSYMSIYAGMV 200
201 FCEAKINDETYQSIMIVVVVGYRIYDVILSPPHIEISLAGEKLVLNCTA 250
251 RTELEVGLOFTWHSSPPSKSHHKVIRNDVKPKPGTVAMKFLSTLTIESVT 300
301 KSDQGEYTCVASSGRMIKRNRTTVRVTKPFIAFGSGKMSLVEATVGSQV 350
351 RIPVKYLSPYAPDIKMYRNGRPIESNYTMIVGDELTEMVTERDAGNYTV 400
401 ILTNP1SMEKQSHMVSLLVNVPPQIGEKAISPMDSYQYGTMTLTCVY 450
451 ANPPLMHHIQWYQWLLEACSYRPGQTSPYACKEWHRVEDFOQQGNKIEVTQ 500
501 QYALIPEGKNTVSTVLIQAAANVSALYKCEAINKAGRGERVISPVIRGP 550
551 ITVQPAQPTEQESVSLLCTADRNTFENLTWYKLGCSQATSVHMGESLTPV 600
601 CKNLDALWKLNGMTFSNSTNDILIVAFQNALSQDGDVYCSAQDKTKR 650
651 HCLVKQLIILERMAPMITGNLENQTTIGETIEVTCPSAGNPTPHITWFK 700
701 DNTELVEDGICVLRDGNRNLTIIRRVRKEDGGLYTCQACNVLGCARAETLF 750
751 IIEGAQEKTNLLEVILVGTAVIAMFFWLLLIVLRLTVKRANEGETLKGYL 800
801 SIVMDPDELPLDERCERLPFYDASKWEFPDRRLXLGKPLGRGAPGVIEAD 850
851 AFGIDKTATCKTVAKMLKEGATHSEHRAIMSELKILIHIGHHLNNVNL 900
901 GACTKPGGPLMVIVEFCKFGNLSTYLRGKRNEFVPYKSKGARPROGKDYV 950
951 GELSVULKRRLOSITSSQSSASSGFVVEKSLSDVEEEEASEEELYKDFLTL 1000
1001 EHLICYSFCVAKGMEMFLASRKCIHDLAARNILLSEKNNVVICDFGLARD 1050
1051 IYKDPDVVRKGDLRPLKWMAPETIFDRVYTIQSDVNSPGVLLWEIPSL 1100
1101 GASPYPGVKIDEFCRRLKEGTRMRAPDYYTPENYQTMLOCWHEDPNQR 1150
1151 PSFSELVEHGNLHQANAQQDGKDYIVLPMSETLSMEEDSGLSLPTSPV 1200
1201 SCMEEEEVCDPKHYDNTAGISHYQLNSKRKSRSRVSVKTFEDIPLPEPB 1250
1251 VRVIPDDSGMVLASEELKTLLEDNLKLSPSFCOMMPSKSRESVASE 1300
1301 GSNTQTCGYSGYHSDDTDTVYSSDEAGLLXNMVDAAVHADSGTTLQLTS 1350
1351 CLNGSGPVPAPPPTPGNHERGAA 1373

(57) Abstract: The invention relates to novel compounds that act to prevent dimerisation of vascular endothelial growth factor (VEGF) receptors. The novel compounds may comprise the amino acid sequence of the fourth Ig-like domain of a VEGF receptor, or a variant that retains the ability to bind to a VEGF receptor. These compounds are useful in the inhibition of the biological activity of VEGF receptors and may thus be used to treat diseases in which VEGF plays a role.

WO 01/42284 A3



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A. CLASSIFICATION OF SUBJECT MATTER					
IPC 7	C12N15/12	C12N15/10	C12N15/62	C12N15/66	C07K14/71
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TANAKA KEIJI ET AL: "Characterization of the extracellular domain in vascular endothelial growth factor receptor-1 (Flt-1 tyrosine kinase)." JAPANESE JOURNAL OF CANCER RESEARCH, vol. 88, no. 9, 1997, pages 867-876, XP001015447 ISSN: 0910-5050 page 875, left-hand column, paragraph 3 --- -/-	15, 17-19, 22-24

Further documents are listed in the continuation of box C.

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHINKAI AKEO ET AL: "Mapping of the sites involved in ligand association and dissociation at the extracellular domain of the kinase insert domain-containing receptor for vascular endothelial growth factor" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, US, vol. 273, no. 47, 20 November 1998 (1998-11-20), pages 31283-31288, XP002166839 ISSN: 0021-9258 figure 1 ---	15, 17-19, 22-24
X	KENDALL R L ET AL: "IDENTIFICATION OF A NATURAL SOLUBLE FORM OF THE VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR, FLT-1, AND ITS HETERO DIMERIZATION WITH KDR" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, US, ACADEMIC PRESS INC. ORLANDO, FL, vol. 226, no. 2, 13 September 1996 (1996-09-13), pages 324-328, XP000611908 ISSN: 0006-291X cited in the application the whole document ---	15, 17-19, 22-24
X	WO 94 21679 A (KENDALL RICHARD L ; THOMAS KENNETH A JR (US); MERCK & CO INC (US)) 29 September 1994 (1994-09-29) figure 1 ---	15, 17-19, 22-24
Y	BARLEON BERNHARD ET AL: "Mapping of the sites for ligand binding and receptor dimerization at the extracellular domain of the vascular endothelial growth factor receptor FLT-1." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 16, 1997, pages 10382-10388, XP002174230 ISSN: 0021-9258 cited in the application the whole document ---	1-29 -/-

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/04693

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KENDALL ET AL: "Inhibition of vascular endothelial cell growth factor by an endogenously encoded soluble receptor" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, US, vol. 90, no. 22, 15 November 1993 (1993-11-15), pages 10705-10709, XP002146190 ISSN: 0027-8424 cited in the application the whole document ----	1-29
A	WO 97 44453 A (CHEN HELEN HSIFEI ;FERRARA NAPOLEONE (US); GENENTECH INC (US); PRE) 27 November 1997 (1997-11-27) the whole document ----	
A	WO 95 33050 A (SHARKEY ANDREW MARK ;SMITH STEPHEN KEVIN (GB); LYNXVALE LTD (GB);) 7 December 1995 (1995-12-07) the whole document ----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No	
PCT/GB 00/04693	

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9421679 A	29-09-1994	AU 684498 B AU 6393494 A CA 2158745 A EP 0694042 A JP 8508161 T US 5712380 A US 5861484 A		18-12-1997 11-10-1994 29-09-1994 31-01-1996 03-09-1996 27-01-1998 19-01-1999
WO 9744453 A	27-11-1997	US 6100071 A AU 717112 B AU 3060497 A EP 0907733 A JP 2000502357 T NZ 332779 A US 5952199 A		08-08-2000 16-03-2000 09-12-1997 14-04-1999 29-02-2000 29-06-1999 14-09-1999
WO 9533050 A	07-12-1995	AU 695393 B AU 2535495 A CA 2191071 A CN 1159827 A EP 0783571 A JP 10504183 T NZ 285834 A US 6011003 A		13-08-1998 21-12-1995 07-12-1995 17-09-1997 16-07-1997 28-04-1998 28-10-1998 04-01-2000